THE UNIVERSITY
of EDINBURGH

Mechanisms of action of 5α-tetrahydrocorticosterone, a novel anti-inflammatory glucocorticoid

Annalisa Gastaldello

Doctor of Philosophy

The University of Edinburgh

2015

Abstract

Topical glucocorticoids (GCs), such as hydrocortisone (HC), are the main drugs used to treat inflammatory skin conditions including eczema and psoriasis, but their long-term use is limited by the onset of side effects such as skin thinning, impairment of wound healing and systemic metabolic dysfunction. For this reason, there is a substantial need for new compounds with the same anti-inflammatory effects but fewer adverse effects. Previous studies have suggested 5α-tetrahydrocorticosterone (5α-THB) as a new, more selective anti-inflammatory compound; this steroid is a metabolite of the major endogenous GC in rodents, corticosterone (B). Preliminary data indicated that 5α-THB may be as effective as HC in reducing mouse irritant dermatitis, but without the local or systemic side effects of HC. The aim of this thesis is to investigate the mechanisms through which 5α-THB delivers more selective anti-inflammatory effects, with the hypothesis that 5α-THB influences distinct signalling pathways from those of B.

A mouse model of irritant dermatitis induced by topical application of croton oil on the ear was developed, and the anti-inflammatory properties of 5α-THB were analysed, in comparison with those of B, after 6 and 24 hours of treatment. In inflamed tissue, B reduced tissue oedema and cell infiltration at both time points; in contrast, 5α-THB did so at 24 but not 6 hours, at a dose five-fold higher than B. Real-time analysis at 24 hours showed that B and 5α-THB similarly reduced the croton oil-induced increase of transcripts of genes encoding vascular and cellular adhesion molecules. Interestingly, while B did not affect the abundance of transcripts of the anti-inflammatory gene *Dusp1*, 5α-THB increased it in croton oil-treated ears, suggesting a different mechanism of action between 5α-THB and B. The experiment was repeated with the injection of the glucocorticoid receptor (GR) antagonist RU486; RU486 relieved the effect of B on swelling but did not attenuate the anti-inflammatory effects of 5α-THB, indicating a further important difference between the two steroids.

Angiogenesis is fundamental for the healing process, and it is known that topical GCs impair wound healing in part by inhibiting angiogenesis; for this reason, the effects of 5α-THB on the formation of new vessels, in comparison with B, were tested in a mouse model of inflammatory angiogenesis induced by sub-cutaneous implantation of polyurethane sponges. 5α-THB, at equipotent doses to B for the reduction of macrophage infiltration, inhibited angiogenesis to a lesser extent than its precursor. In addition, B had systemic effects in that it lowered adrenal gland weights, whereas 5α-THB did not. Histological analysis suggested that while B inhibits formation and maturation of new vessels, 5α-THB may affect only the former process. Molecular analysis showed that B reduced the abundance of transcripts of the majority of the tested genes involved in inflammation, angiogenesis and tissue remodelling, but 5α-THB had more selective effects.

Ex vivo studies in mouse bone marrow-derived macrophages stimulated with LPS showed that 5α-THB inhibited release of pro-inflammatory cytokines in a weaker manner compared with B. This inhibition was partially prevented by co-incubation of RU486 with B but not with 5α-THB. In *in vitro* studies, molecular pathways activated by B and associated with adverse side effects were only weakly activated by 5α-THB. In particular, 5α-THB only weakly induced phosphorylation of GR, and activation of expression of GC-responsive reporter plasmids and endogenous metabolic genes. Interestingly, 5α-THB reduced B-induced trans-activation of some of these genes.

In summary, 5α-THB effectively reduces skin inflammation, but, unlike B, has only moderate anti-angiogenic properties, and weakly activates molecular mechanisms associated with adverse metabolic side effects. Most importantly, its action may not be due to activation of GR. This work opens the intriguing possibility that GCs work through mechanisms not yet investigated, and this may be of pivotal importance in the search for new safer anti-inflammatory compounds.

Declaration

I declare that this thesis is the result of my own work performed at The University of Edinburgh. Any assistance received has been acknowledged in the relevant sections.

I declare that this work has not been previously submitted for any other degree or qualification.

Annalisa Gastaldello

Acknowledgements

I am grateful to my supervisors Ruth Andrew, Paddy Hadoke and Brian Walker for all their instruction, guidance and feedback throughout my PhD.

Thank you to all the people in CVS for their support; in particular, I am greatly indebted to Dawn for sharing her infinite knowledge and providing practical help whenever I needed.

I am grateful to my office mates, past and present: James, Ruth, Tracy, Mark, Abdullah, Anjie, Anna and Tiina for putting up with my Italian idiosyncrasies. To my MSc and PhD colleagues, thanks a lot for sharing this experience with kindness, compassion and humour. In particular, big hugs go to Diego and Giorgos for all the laughter, great conversations and deep thoughts about life. My PhD would not have been the same without you.

I would also like to extend a big thank you to my far-away friends: Danilo, Alessandro and Cecilia. To Danilo, I am so grateful for all the phone calls that kept me company and made me laugh over the past four years; to Alessandro, for sharing with me your life in Spain; and to Cecilia, your energy, humour and cheekiness are always with me… you make my trips home even more worthwhile.

Limitless thanks go to Nick for his never-ending encouragement and love, and for constantly inciting me to let go.

Most of all, I would like to thank my family. *Soprattutto, grazie alla mia famiglia. Ad Alessandra, ti sarò sempre grata per la tua profonda comprensione; a Daniel, grazie per il tuo senso dell'umorismo e il tuo affetto; a Daniel e Maria, grazie per avermi fatto diventare zia e avermi regalato due bellissimi motivi in più per venire a casa, Emma e Leonardo. A Mamma e Papà, grazie di cuore per aver accettato la lontanza e avermi dato le opportunità che voi non avete mai avuto.*

Contents

List of Figures

List of Tables

Abbreviations

5α-R	5α-reductase
5α-THB	5α-tetrahydro corticosterone
ACTA2	Alpha actin 2
AF	Activation function
AP	Activator protein
B	Corticosterone
bp	Base pair
BSA	Bovine serum albumin
CDH5	Cadherin 5
cDNA	Complementary DNA
Col	Collagen
CRH	Corticotrophin-releasing hormone
DBD	DNA-binding domain
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DUSP1	Dual specificity protein phosphatase 1
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FKBP51	FK506 binding protein 51
GC/GCs	Glucocorticoid/s
GGT1	Gamma-glutamyltransferase 1
GILZ	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRE1/2s	Glucocorticoid response element half sites
GRU	Glucocorticoid responsive unit
h	Hour

H_2O_2	Hydrogen peroxide
HEK293	Human embryonic kidney cell line
HSD	Hydroxysteroid dehydrogenase
HSP	Heat shock protein
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IGFBP	Insulin-like growth factor-binding protein 1
IL	Interleukin
INF	Interferon
IκB	Inhibitor of NF-κB
JNK	c-Jun N-terminal kinases
KD	Knocked down
kDa	kilo Dalton
KO	Knock-out
LBD	Ligand-binding domain
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MCP-1	Monocyte chemoattractant protein-1
MKP	MAPK phosphatase
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MW	Molecular weight
NaOH	Sodium hydroxide
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
PNMT	Phenylethanolamine N-methyltransferase
POMC	Pro-opiomelanocortin

RT-PCR	Reverse transcriptase polymerase chain reaction
STAT	Signal transducer and activator of transcription
TAT	Tyrosine aminotransferase
TAT	Tyrosine aminotransferase
THBS	Thrombospondin
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tris	Trisma base
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WT	Wild-type
αSMA	Alpha smooth muscle actin

List of publications

Abstracts

Orals:

- Gastaldello A, Nixon M, Yang C, Saunders P, Walker B, Chapman K, Andrew R (2013). Understanding the molecular mechanisms underlying the anti-inflammatory properties of 5α-THB. *Program of Scottish Society for Experimental Medicine 2013.*

- Gastaldello A, Nixon M, Yang C, Saunders P, Walker B, Chapman K, Andrew R (2013). Investigation into the molecular mechanisms underlying the anti-inflammatory properties of 5α-THB. *Program of the 95th Meeting of the Endocrine Society, OR03-5.*

Poster:

- Gastaldello A, Nixon M, Yang C, Saunders P, Walker B, Chapman K, Andrew R (2013). Investigation into the molecular mechanisms underlying the anti-inflammatory properties of 5α-THB. *Endocrine Abstracts 31 P317.*

Ring the bells that still can ring
Forget your perfect offering
There is a crack in everything
That's how the light gets in.

From "Anthem", Leonard Cohen

Chapter 1

INTRODUCTION

Chapter 1: Introduction

1.1 Glucocorticoids

1.1.1 General considerations

Glucocorticoids (GCs) are steroid hormones produced by the adrenal cortex, and the name glucocorticoid (**gluco**se + **cort**ex + ster**oid**) derives from their role in the regulation of the metabolism of glucose, their synthesis in the adrenal glands and their steroidal structure. They are necessary for the maintenance of the body's homeostasis, and their production is controlled by the hypothalamic-pituitary-adrenal (HPA) axis in a circadian and ultradian manner; furthermore, their synthesis and release is increased in response to a variety of emotional or physical stress (Oakley and Cidlowski 2013). GCs have a wide range of functions, including the regulation of metabolism, cell growth and differentiation; they also affect mood and cognitive function and exert potent anti-inflammatory and immunosuppressive actions (Blum and Maser 2003; Ismaili and Garabedian 2004). Because of their role in suppression of the inflammatory response, synthetic derivatives of GCs, such as prednisone/prednisolone, dexamethasone and budesonide, are among the most effective anti-inflammatory drugs in use and they are widely prescribed to treat acute and chronic inflammatory diseases, autoimmune diseases, organ transplant rejection and malignancies of the lymphoid system (McDonough, Curtis et al. 2008; Revollo and Cidlowski 2009; Oakley and Cidlowski 2010; Oakley and Cidlowski 2013). In addition, their anti-proliferative and anti-angiogenic properties have also been exploited for the treatment of cancer (Vilasco, Communal et al. 2011; Oakley and Cidlowski 2013). Unfortunately, due to their broad range of physiological actions, the therapeutic benefit of glucocorticoids is limited by severe side effects such as osteoporosis, abdominal obesity, glaucoma, growth retardation in children, hypertension, muscle wasting, impairment of wound healing and skin thinning (Walker 2007; Lowenberg, Stahn et al. 2008; Oakley and Cidlowski 2010). An aim of the pharmaceutical industry is to develop safer drugs with anti-inflammatory effects and, at the same time, fewer side effects.

1.1.2 Glucocorticoid structure and synthesis

Glucocorticoids have a common structure made up of 21 carbon atoms, and each carbon is numbered in a standard way. Like other steroids, they are derived from the cyclopentanoperhydrophenanthrene structure which is formed by three cyclohexane rings and a single cyclopentane ring, labelled A, B C and D respectively (Figure 1.1). GCs, together with mineralocorticoids and some sex steroids, belong to the group of hormones called adrenocorticosteroids; they are synthesized by and released from the zona fasciculata of the adrenal cortex. They derive from the same precursor, the lipid cholesterol, which is produced by the adrenal gland or taken from the blood stream. Cortisol (F) is the main active GC in humans, while in rodents it is corticosterone (B) since the enzyme 17α-hydroxylase is not present in the adrenal gland (Luu-The, Pelletier et al. 2005). Pathways involved in the synthesis of F, B and the main mineralocorticoid hormone aldosterone, are shown in Figure 1.2 and take place in mitochondria. Their synthesis begins with the conversion of cholesterol to pregnenolone by the enzyme 20, 22-desmolase located in the inner mitochondrial membrane; subsequently, reactions of dehydrogenation and hydroxylation take place and form deoxycorticosterone (DOC) through the intermediate production of progesterone. In the zona fasciculata of the adrenal cortex, some of the progesterone is hydroxylated via the action of 17α-hydroxylase and therefore converted to cortisol. In humans, both the zona fasciculata and glomerulosa can convert DOC to B; formation of aldosterone from B occurs exclusively in the zona glomerulosa thanks to the presence of the enzyme aldosterone synthase (Freel and Connell 2004).

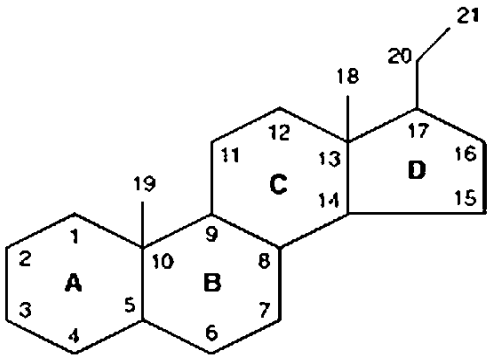

Figure 1.1 Structure of glucocorticoids. A, B and C, cyclohexane rings; D, cyclopentane ring; 1-21, standard numbering for the carbon atoms.

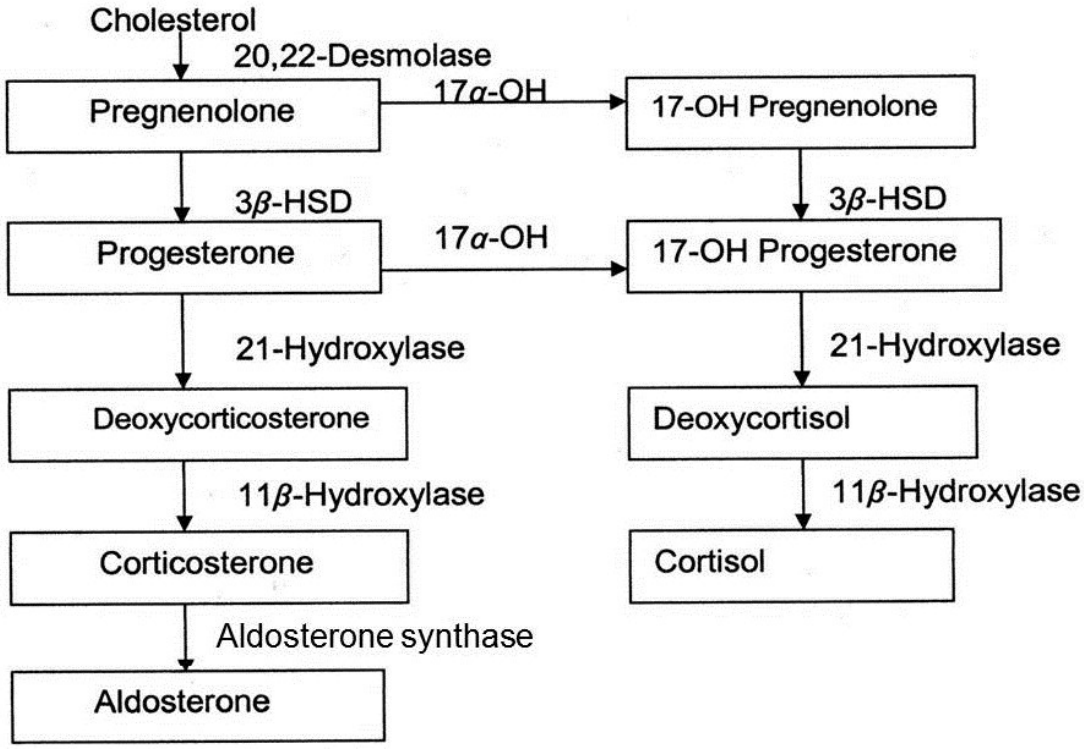

Figure 1.2 Common pathways of synthesis of glucocorticoids and aldosterone. 17α-OH = 17α-hydroxylase, 3β-HSD = 3β-hydroxysteroid dehydrogenase. Adapted from (Freel and Connell 2004).

1.2 The glucocorticoid receptor (GR)

Natural and synthetic GCs exert their actions via the glucocorticoid receptor (GR), a steroid hormone receptor that belongs to the nuclear receptor superfamily of transcription factors. Consistent with the wide-ranging effects of GCs, the receptor is ubiquitously expressed and necessary for life (Cole, Blendy et al. 1995). GCs can also bind the mineralocorticoid receptor (MR) with a similar affinity to aldosterone; however, the presence of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD 2) in tissues targeted by aldosterone (such as the kidney) prevents the binding of GCs to MR by converting the active form (F or B) into the inactive one (cortisone or 11-dehydrocorticosterone) (Seckl and Walker 2001).

1.2.1 GR isoforms

GR is derived from a single gene (*NR3C1*) (Evans 1988) found in the chromosome 5 in humans and composed of nine exons, but alternative splicing gives rise to different

isoforms (Figure 1.3, page 30) The exons 2-9 represent the protein-coding exons, whilst exon 1 is the 5' untranslated region; exons 2-8 are always present in the GR messenger RNA (mRNA), while the exon 9 has two isoforms, α and β, alternative splicing of which give rise to two mature isoforms of transcripts: splicing of exon 9α produces the major GR isoform, GRα, which is translated to generate a protein with a unique sequence of 50 amino acids (aa) at its C-terminus; splicing of exon 9β gives rise to the variant GRβ, which is translated to assemble a protein with a distinct 15 aa-long tail at the C-terminus. GRα (777 aa-long) is the main active isoform as it binds GCs, it translocates to the nucleus and it regulates gene expression (Rhen and Cidlowski 2005; Oakley and Cidlowski 2013). GRβ (742 aa-long) does not bind GCs, and it seems to function as an inhibitor of GRα; however, in recent years, evidence has been accumulated in favour of a role of this isoform in regulating directly gene expression; in effect, despite agonists not being reported to bind GRβ, the antagonist RU486 does bind this GR isoform and regulates its transcriptional activity (Lewis-Tuffin, Jewell et al. 2007; Kelly, Bowen et al. 2008; Kim, Oh et al. 2008; Kino, Manoli et al. 2009). Other minor isoforms derived from alternative splicing are also present, for example GRγ, which contains an insertion of a single arginine, binds GCs and DNA to a similar extent to GRα but its gene regulation profile is different. Its expression is associated with glucocorticoid resistance in many cancers (Thomas-Chollier, Watson et al. 2013). Two others are GR-A and GR-P which lack the region of the receptor responsible for ligand binding and, as a consequence, lack the ability to bind GCs. While little is known about GR-A, GR-P seems to be the main variant expressed in GC-insensitive cancers (Gaitan, DeBold et al. 1995; Krett, Pillay et al. 1995; de Lange, Segeren et al. 2001). This scenario is made even more complex considering that GRα exists in 8 different isoforms (A, B, C1, C2, C3, D1, D2, D3) produced by alternative translation initiation sites in exon 2; all these isoforms bind GCs and interact with target genes, however GRα-C isoforms are the most biologically active, while GRα-D isoforms are the least (Kadmiel and Cidlowski 2013). It is predicted that 8 translational isoforms may exist also for GRβ (Kino, Su et al. 2009). The above-mentioned diversity in GR transcription and translation give rises to differential responses to GCs; indeed, their

effects also depend on which isoforms are present, and in which proportion, in a particular cell type.

1.2.2 GR localization and structure

In the absence of its ligand, GR resides in the cytoplasm forming a complex with chaperonic molecules composed of heat shock protein Hsp90, 70, 23 and other regulatory factors (Pratt and Toft 2003; Baschant and Tuckermann 2010). Upon ligand binding the chaperonic complex is dissociated and the exposure of specific nuclear localization sequences triggers its translocation to the nucleus, where it regulates gene expression. All members of the nuclear receptor superfamily, including GR, are modular proteins composed of an N-terminal trans-activation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). Moreover, a flexible region called the "hinge region" connects the DBD and LBD (Figure 1.3). The most conserved region in the family is the DBD that contains two zinc finger motifs that recognize and bind DNA sequences, called glucocorticoid-responsive elements (GREs). The NTD domain possesses a strong transcriptional activation function (AF1) which binds several co-regulators and components of the transcription machinery. The LBD forms a hydrophobic pocket for binding glucocorticoids and it consists of 12 α-helices and four β-sheets (Bledsoe, Montana et al. 2002). Another activation function that interacts with co-regulators in a ligand-dependent way is present in the LBD; moreover, two nuclear localization signals termed NL1 and NL2 are localised at the border DBD/hinge region and within the LBD respectively.

1.2.3 Post-translational modification of GR

Functional diversity in GR activity is achieved by alternative splicing taking place at the transcriptional level, as mentioned above, but also by modifications occurring at post-translation level. Among the latter, phosphorylation of serine and threonine residues is one of the most important in determining GR activity, influencing nuclear translocation and GR degradation. The most characterised GR phosphorylation sites in humans are serines (S113, S134, S141, S143, S203, S211, S226 and S404). Some of these sites (i.e. S211) are phosphorylated in a ligand-dependent manner, while for

others this modification is ligand-independent; indeed, it has been recently demonstrated that S134 is phosphorylated by p38 MAPK in a hormone-independent fashion (Rhen and Cidlowski 2005; Galliher-Beckley, Williams et al. 2011). In the past few years it has become clear that the outcome of GR phosphorylation in terms of gene expression is residue- and cell type-dependent, and it is likely due to the fact that phosphorylation of different sites induces differential conformation changes and recruitment of co-activators (Webster, Jewell et al. 1997; Wang, Frederick et al. 2002). In effect, phosphorylation of sites such as S211 and S203 has been demonstrated to stimulate the GC-induced up-regulation of target genes, while phosphorylation of S226 and S404 has been shown to inhibit it. One example of the former is the induction by GCs of the gene *Igfbp1*, which is dependent on phosphorylation of S211 and consequent recruitment of the co-activator MED14 (Poolman, Farrow et al. 2013). Other types of post-translational modifications are binding of ubiquitin induced by GCs such as cortisol, which promotes the degradation of GR via the proteasome, and sumoylation which refers to the attachment of small molecules similar to ubiquitin (Rhen and Cidlowski 2005). Recent studies suggest that the latter increases the transcriptional activity of GR (Le Drean, Mincheneau et al. 2002; Holmstrom, Van Antwerp et al. 2003). Furthermore, acetylation has also been reported on lysines 494 and 495 with consequent impairment of the ability of GR to inhibit the action of the transcription factor NF-κB (Barnes 2009). In figure 1.3 a simplified representation of the characteristics and processes described above is given.

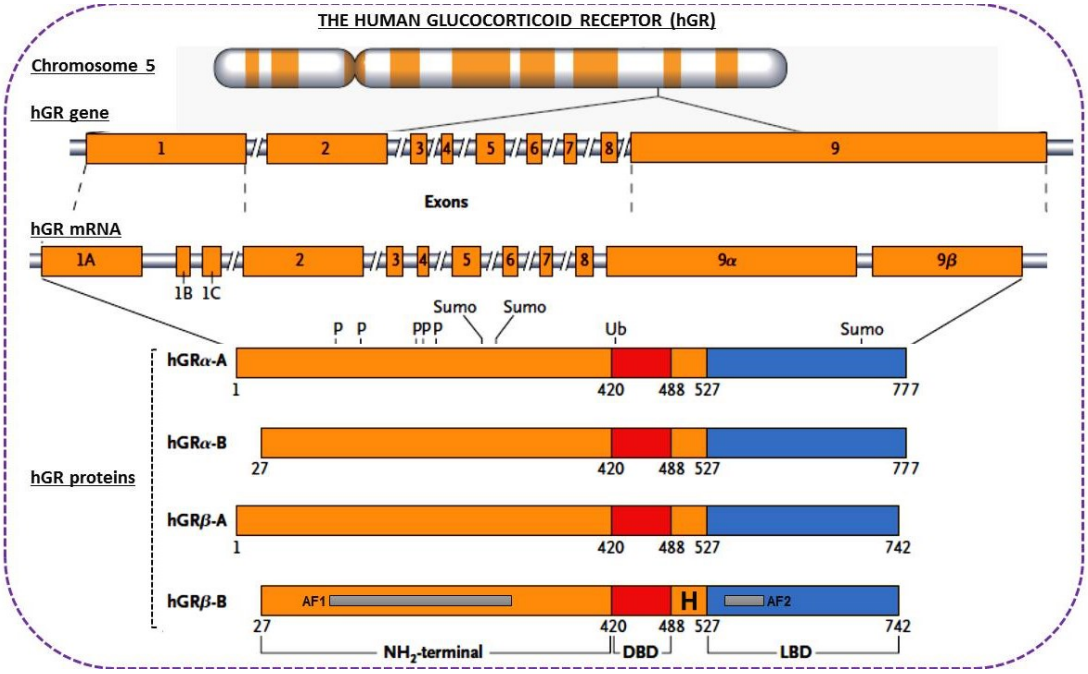

Figure 1.3 Schematic representation of the transcriptional origin of the human glucocorticoid receptor (hGR), its isoforms and modular structure. DBD = DNA-binding domain; H = hinge region; LBD = ligand-binding domain; AF = activation function; P, Ub, Sumo = phosphorylation, ubiquitinilation and sumoylation sites; mRNA = messenger RNA. In the representations of the hGR gene and mRNA numbers identify exons, while in hGR protein they refer to the amino-acidic residues. Adapted from (Rhen and Cidlowski 2005).

1.2.4 GR signalling

GR modulates gene expression and cellular behaviour via several genomic and non-genomic mechanisms. The former refers to modulation of gene expression by interaction of GR with DNA, and is dependent on GR translocation to the nucleus and is observable within 15-150 min from cellular exposure to GCs (Haller, Mikics et al. 2008). The classical model is based on binding of a hormone-bound GR dimer to GREs in the regulatory regions of target genes. However, in spite of the fact that GRs are expressed in most tissues, the set of regulated genes differs in each tissue; this observation gave rise to the necessity for a revision of this classical model, and to the discovery of different mechanisms through which GR achieves selective,

differential control of gene expression, including recruitment of co-regulators and chromatin remodelling (Biddie 2011; Biddie, John et al. 2011).

1.2.4.1 Activation of gene transcription

Ligand-bound GR can activate gene transcription by direct binding to so-called simple GRE. Based on studies of a number of GREs, a consensus GRE has been defined as a 15-bp imperfect palindrome, 5'-GGTACAnnnTGTTCT-3' (Nordeen, Suh et al. 1990), in which the 3' half is the most conserved and the 5' one is highly variable (Barnes 1998). The base positions are numbered as -6,-5,-4,-3,-2,-1,nnn,1,2,3,4,5,6, where n is any nucleotide; it has been shown that only certain nucleotides are critical, and these are found at position -3, -2, +2, +3 and +5. GR forms dimers as two GR monomers bind in turn to the 3' and then 5' half sites allowing interactions of the dimerisation domains in the DBD (Tsai, Carlstedt-Duke et al. 1988; Dahlman-Wright, Siltala-Roos et al. 1990; Hard, Dahlman et al. 1990; La Baer and Yamamoto 1994). The 3-bp spacer between the two half-sites is a requirement for cooperative binding of the two monomers (Dahlman-Wright, Wright et al. 1991). Such simple GREs were found in a number of genes, such as Serine/Threonine protein kinase (*Sgk1*) and β2-adrenergic receptor, and they are a good match to the consensus. However, GC induction of these genes is only moderate. In several genes, binding of GR to GREs is not sufficient, and recruitment of other transcription factors (TFs) to adjacent binding sites is required. These composite regulatory sequences are often referred to as glucocorticoid responsive units (GRUs), as the different elements are spatially and functionally clustered. These units differ as the identity of recruited TFs varies in a cell and gene-specific manner (De Bosscher, Beck et al.; So, Chaivorapol et al. 2007; Baschant and Tuckermann 2010), and also in the number of elements in each unit, ranging from 4 to 10. Examples of TFs are C/EBP, FoxA, CBP/p300, P/CAF and steroid receptor co-activator (SRC)-1 (Smoak and Cidlowski 2004). In contrast to simple GREs, induction of genes can be increased up to 23-fold in response to GCs, as in the case of the carbamoylphosphate-synthetase (CPS) gene. More modest induction is obtained for metabolic genes such as Phenylethanolamine N-Methyltransferase

(*Pnmt*), tyrosine aminotransferase (*Tat*) and phosphoenol pyruvate carboxykinase (*Pepck*) in the liver.

GR can also bind the DNA as a monomer at so-called GRE half-sites (GRE1/2) (Segard-Maurel, Rajkowski et al. 1996); however, additional elements are required to mediate a glucocorticoid response. These elements may be represented by other TFs, by multiple GRE1/2s or classical GRE. An example of the first case is the induction of the phenylalanine hydroxylase gene, while for the activation of the hCYP3A gene, GR binds to two GRE1/2s which enable GCs responsiveness. In the mouse mammary tumour virus (MMTV) there are four GRE1/2s, and GR binds as homodimer to the two proximal ones. Interestingly, it is suggested that this kind of dimerisation, happening on directly repeated GRE1/2s, is mediated by the dimerisation function found in the LBD (Aumais, Lee et al. 1996). In the case of the thyrotropin-releasing hormone gene, however, a combination of a GRE and a GRE1/2 operates (Hovring, Matre et al. 1999).

Induction of transcription by GR mainly targets genes controlling metabolism (e.g. gluconeogenesis), however, this mechanism also explains the increased expression of anti-inflammatory genes. An example is induction of the anti-inflammatory cytokine IL-10. It is known that GR binds as a monomer to the transcription factor STAT3 (Signal Transducer and Activator of Transcription 3) increasing its transcriptional activity to promote expression of IL-10 in human B cells (Unterberger, Staples et al. 2008), while transcription of the anti-inflammatory protein IkBα results from GR dimers interacting with specific sequences within its promoter. In addition, studies have shown that GCs can modulate the rate of degradation of mRNAs through trans-activation of key genes involved in the process, therefore decreasing the expression of pro-inflammatory cytokines in an indirect manner; for example, in the case of TNFα there is some evidence that GR may act by inducing expression of the protein tristetraprolin (TTP), which in turn causes TNFα mRNA destabilization and degradation (Smoak and Cidlowski 2004; Clark 2007).

1.2.4.2 Repression of gene transcription

GR is also able to inhibit transcription, and this behaviour has long been regarded as the reason behind the majority of the anti-inflammatory properties of GCs. The main mechanism involved is called "trans-repression" where GR hinders the action of pro-inflammatory DNA-bound transcription factors, such as Nuclear Factor-kB (NF-κB) and Activator Protein-1 (AP-1), thus preventing transcription of pro-inflammatory genes. NF-κB is a complex of several units, in which the heterodimer formed by the proteins p65/p60 is the predominant. GR binds to the p65 subunit resulting in the inhibition of gene transcription mediated by NF-κB. AP-1 instead, is formed by a member of the Jun family (c-Jun, v-Jun, Jun-B or Jun-D) homodimerised with another member from the same family or heterodimerised with a Fos protein (c-Fos, Fos-B, Fra-1 or Fra-1), and GR interacts with the Jun member inhibiting its action to promote pro-inflammatory gene transcription (Smoak and Cidlowski 2004). Another trans-repression mechanism involves GR competing with co-activators of NF-κB, such as cAMP response element binding protein (CREB)-binding protein (CBP) and steroid receptor co-activator (SRC)-1, required for NF-κB maximal activity (Schoneveld, Gaemers et al. 2004); furthermore, GR has been reported to recruit co-repressors, for example GRIP, and this represents another mechanism whereby the receptor can down-regulate gene expression (Uhlenhaut, Barish et al. 2013). Another way in which GR inhibits transcription is by binding to so-called negative GREs (nGREs) which harbour sequences more variable than those of classical positive GREs (Schoneveld, Gaemers et al. 2004). Examples of this mechanism are inhibition of genes such as the Corticotropin Releasing Hormone (CRH), ProOpioMelanoCortin (POMC) and osteocalcin, where GR binds mainly as monomer on the promoter of the former, and as trimer on the latter (Drouin, Sun et al. 1993). This mechanism is associated with some of GC-induced side effects.

While this description of how GR can interact with the DNA and regulate gene expression is based on experimental data, the story might be much more complex. Indeed, a recent paper claims that while up to 20% of GR-mediated repression is accounted for by GR found at tethered and nGREs sites in lipopolysaccharide (LPS)-stimulated macrophages, around 20% of GR-induced genes contain the same DNA

motifs; this makes predictions about GR effects on gene expression based on the classification of these motifs unreliable (Uhlenhaut, Barish et al. 2013). As our knowledge of the DNA sequences targeted by GR deepens, an even more complex scenario is emerging, and this scenario seems to suggest that GR itself may not be the central player when it comes to determining the fate of its own actions. Indeed, GR may be more of a co-actor than the protagonist of what happens at gene level; for instance, epigenetic regulators and chromatin context may be other players as important as GR itself (Uhlenhaut, Barish et al. 2013).

1.2.4.3 Non-genomic glucocorticoid actions

GR also regulates cell physiology via non-genomic mechanisms, which are mediated by cell signalling proteins and do not involve gene transcription (Stojadinovic, Sawaya et al. 2013). While genomic actions of GR are relatively slow, the non-genomic effects take place in a shorter time frame, within 5 minutes or even seconds from cell exposure to GCs (Haller, Mikics et al. 2008). Among them, the interaction of GR with signalling pathways such as JNK and the T cell receptor signalling complex, may explain the modulation of pro-inflammatory gene expression (Baschant and Tuckermann 2010), while the release of chaperone proteins, such as SRC, upon ligand binding, are shown to contribute to other rapid glucocorticoid effects (Croxtall, Choudhury et al. 2000). Indeed, SRC interacts with lipids in the plasma membrane blocking the production of one of the precursors to inflammatory molecules (prostaglandin and leukotrienes), namely arachidonic acid (Croxtall, Choudhury et al. 2000); this is therefore a non-genomic mechanism that contributes to the anti-inflammatory effects of GCs (Alangari 2010). Some other signalling pathways influenced by GCs are those involving MAPKs, cAMP-PKA, PI3K and PLC-PKC proteins, in a variety of tissues such as neuronal and erythroid cells (Croxtall, Choudhury et al. 2000; Song and Buttgereit 2006; Stellacci, Di Noia et al. 2009; Stojadinovic, Sawaya et al. 2013). Some other non-genomic effects are thought to be mediated by interactions with G protein-coupled receptors (GPCRs) and the membranous GR (mGR), distinct from the classical cytosolic GR (Orchinik, Murray et al. 1991; Gametchu, Chen et al. 1999; Bartholome, Spies et al. 2004; Song and Buttgereit 2006; Roozendaal, Hernandez et al. 2010; Stojadinovic, Sawaya et al.

2013). Furthermore, GCs have been shown to bind other receptors associated with membranes such as the gamma-aminobutyric acid (GABA) receptors (Majewska 1987) and the low affinity glucocorticoid receptors (LAGs) (Roszak, Lefebvre et al. 1990). GCs also alter the fluidity of the plasma and mitochondria membrane by interacting with lipids. At high concentrations they have been shown to be incorporated into membranes and, as a consequence, to influence the activity of proteins associated with them, such as cation channels (Stahn, Lowenberg et al. 2007); these effects may explain in part the anti-inflammatory properties of GCs in that they may lead to inhibition of production of ATP (Song and Buttgereit 2006).

1.3 Glucocorticoid metabolism

The concentration of circulating and local GCs within tissues, and reaching GR, is regulated by several mechanisms. The release into the blood stream and the availability of GCs is controlled mainly by the HPA axis and by the corticosteroid-binding globulin (CBG) in serum, while locally, within tissues and cells, the presence of metabolizing enzymes (such as 11β-hydroxysteroid dehydrogenases) is the major factor influencing the ratio between the amount of active and inactive GCs (Kadmiel and Cidlowski 2013).

The principal pathway responsible for the metabolism of glucocorticoids takes place in the liver. Here, F and B, and their inactive forms cortisone and 11-dehydrocorticosterone, are metabolised via A-ring reductions by A-ring reductases, including 5α- and 5β-reductases and 3α-hydroxysteroid dehydrogenase (3α-HSD).

5α- and 5β-reductases catalyse the irreversible *trans* or *cis* reduction of the double bond at position $\Delta^{4,5}$, forming *trans* 5α- and *cis* 5β- dihydro-glucocorticoids. These reactions are followed by another reduction by 3α-HSD to produce 5α- and 5β-tetrahydro metabolites. This two-step A-ring reduction is common to other steroids including aldosterone, progesterone and androgens (e.g. testosterone). As the main interest of this PhD project is the compound 5α-tetrahydrocorticosterone (5α-THB), which is the product of the activity of 5α-reductases, Figure 1.4 gives a representation of these metabolic reactions without including the 5β-metabolites or

enzymes. Furthermore, I will focus my attention principally on describing the characteristics of the 5α-reductase enzymes, and introduce here some important findings regarding mice in which the activity of one of the isoforms of 5α-reductases has been disrupted.

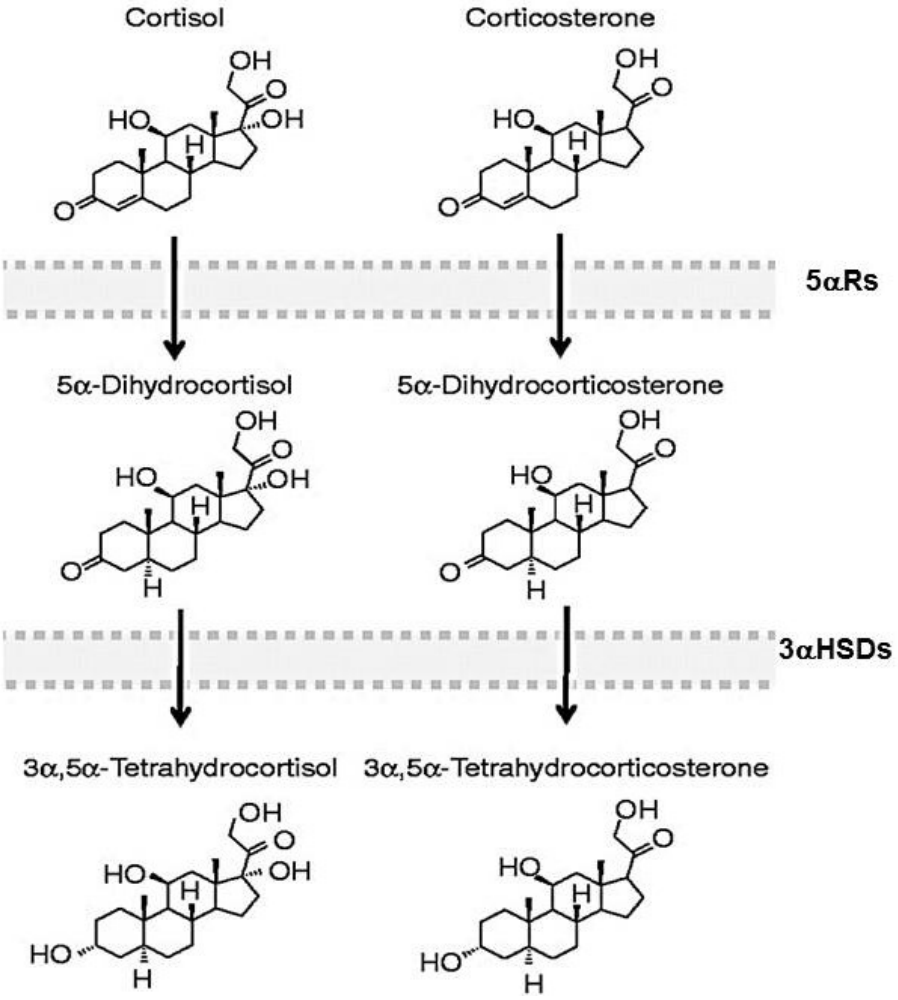

Figure 1.4 The A-ring reduction pathways for cortisol and corticosterone. 5αRs = 5α-reductases, 3αHSDs = 3α-hydroxysteroid dehydrogenase. Adapted from (Nixon, Upreti et al. 2011).

1.3.1 5α-Reductases

There are two isozymes of 5α-reductases that catalyse 5α-reduction of steroids: the type I isozyme (5α-R1) is expressed principally in the liver of mice, and in the liver, kidney and skin of humans; the type II isozyme is expressed principally in the genital

tract, including prostate, of mice, and in the liver, prostate and skin of humans. The genes encoding these enzymes (*SRD5A1* and *SRD5A2*) are members of a larger family of genes containing 5α-reductase domains, one of the other members being encoded by the gene *SRD5A3*, which has a less clear role. Conservation in the C- and N-terminal regions between it and 5α-R1 and 5α-R2 supports a role for resultant SRD5A3 protein in the metabolism of steroids. However, to date, this has not been demonstrated for GCs (Nixon, Upreti et al. 2011).

1.3.2 5α-Reduced glucocorticoids

Many studies have demonstrated that 5α-reduced metabolites of other hormones such as testosterone, aldosterone and progesterone, are potent agonists of the same receptor as their precursor (Siiteri and Wilson 1974; Rapkin, Morgan et al. 1997). Recently, McInnes *et al.* showed that 5α-reduced GCs are able to bind to and activate GR, while the 5β-reduced metabolites are inactive as agonists of GR (McInnes, Kenyon et al. 2004). In addition, there is evidence suggesting that the 5α– and 5β-reduction of GCs is increased in human obesity (Andrew, Phillips et al. 1998), and that 5α-R1 expression and activity is increased in obesity in rodents and humans (Fraser, Ingram et al. 1999; Rask, Olsson et al. 2001; Livingstone, McInnes et al. 2005). As a result, the rates of glucocorticoid inactivation are increased in the liver in obesity and this may be a compensatory protective mechanism ameliorating metabolic risk factors. My supervisors' group recently has shown that mice harbouring a disrupted 5α-R1 enzyme have an adverse metabolic phenotype (Livingstone, Barat et al. 2014). However, although one might expect that increased glucocorticoid action in the liver as a result of 5α-R1 deficiency would reduce susceptibility to intrahepatic inflammation in these mice, 5α-R1 knockout mice had an exaggerated response to liver injury with carbon tetrachloride. The explanation for this disassociation between metabolic and inflammatory effects may lie in the intrinsic properties of the 5α-reduced glucocorticoid product of 5α-R1.

My supervisors' group has shown that the product of the 5α-R1 pathway, 5α-THB, possesses selective actions. Indeed, this compound can bind to GR with equivalent affinity to B (McInnes, Kenyon et al. 2004) and stimulate nuclear translocation of the

receptor (Yang 2009); however, it activates metabolic genes (requiring conventional GR dimerisation) only very weakly *in vitro* and not at all *in vivo*, while retaining the ability to potently suppress cytokine release (believed to be executed via GR monomers) from activated macrophages and also to invoke immune suppression (McInnes, Kenyon et al. 2004; Yang, Nixon et al. 2011). Furthermore, 5α-THB is able to reduce skin inflammation (irritant eczema, also known as dermatitis) induced by croton oil in mice with a potency comparable with that of hydrocortisone (HC); most importantly though, 5α-THB does not cause skin thinning or systemic metabolic changes (e.g. increase of circulating insulin) triggered instead by application of HC (Livingstone, Sykes et al. 2014). Therefore 5α-THB has the properties of an attractive anti-inflammatory drug, and dissection of the distinct pathways activated by B or 5α-THB may provide further opportunities for selective modulation of inflammatory versus metabolic processes.

1.4 The effects of glucocorticoids on the immune system

Inflammation is a vital response of the organism to infection, injury and tissue stress aimed to restore and maintain body homeostasis. In the past few decades it has been recognised that, in addition to already well-characterised inflammatory diseases, for instance asthma, dermatological manifestations (dermatitis, psoriasis) and inflammatory bowel disease just to name a few, a greater number of pathological conditions than previously thought are characterised by persistent and unresolved inflammation, such as obesity, type II diabetes, atherosclerosis and cancer. As a consequence, the quest for anti-inflammatory agents has intensified (Uhlenhaut, Barish et al. 2013). Importantly, this search is characterised by the need to find safer compounds than those available on the market. Among the most effective and prescribed anti-inflammatory drugs in use are synthetic derivatives of GCs. As mentioned above, the anti-inflammatory effects of these compounds are mediated largely, but not exclusively, through regulation of gene expression in a variety of ways; however, to truly understand why they are so powerful and important anti-inflammatory agents, both from a physiological and a clinical point of view, we need to consider how broadly they affect the immune response and its cellular

components. Below, a summary is given of their effects on the different cell types of the immune system.

GCs act by influencing the behaviour of nearly all immune cells (reviewed in Zen, Canova et al. 2011). Regarding their effects on the T cell population, they inhibit the production of the cytokine IL-2 through a mechanism affecting the activity of transcription factors involved in its expression. In addition, they also work through non-genomic mechanisms and inhibit signalling pathways by binding to membrane GR rather than the cytoplasmic form; as an example they block the transduction of important signals through the T-cell receptor (TCR) by inhibiting the protein kinase Scr and causing its dissociation from TCR. Furthermore, they interfere with pathways required for proliferation of T cells by disrupting calcium-dependent signalling cascades. They also induce pro-apoptotic effects in T helper cells 1 and 2 by binding to GC receptors in the mitochondria. However, on a subpopulation of T cells identified as regulatory T cells they have pro-survival effects, highlighting how GCs are not merely inhibitors of the immune response, but more controllers of this complex system that ought to be kept in balance in order to maintain body homeostasis. Despite a lack of intense investigation of the effects of GCs on B cells, it is known that they exert some moderate actions on this population by influencing cell survival, proliferation and activity. For instance, they trigger pro-apoptotic signals by reducing the expression of the anti-apoptotic protein B-cell lymphoma (Bcl)-2. Another example of the complexity of the interaction between the immune system and GCs comes from the study of their effects on the macrophage population. Indeed, low concentrations of GCs stimulate their function by affecting adhesion, chemotaxis, phagocytosis and cytokine production; on the other hand though, high concentrations of GCs exert immunosuppressive effects on the same population, most of all on activated macrophages. They also increase the phagocytic activity of these cells, for example towards neutrophils. Another important group of cells affected by GCs are dendritic cells (DC), which act as messengers between the innate and the adaptive immune system as their main function is to process and present antigenic material to T cells. GCs disturb their maturation and function; for instance, stimulated DC pre-treated with dexamethasone display a reduced ability to activate T

cells, with a consequent decrease in their proliferative response and in the production of interferon (INF). Furthermore, granulocytes are targeted by GCs in that the maturation and the production of cytokines, chemokines and arachidonic acid derivatives are inhibited by these hormones in mast cells. They also inhibit the release of histamine from basophils and reduce their number, and induce apoptosis of eosinophils and neutrophils.

The picture drawn above of the broad spectrum of effects of GCs on the immune system is of course only a simplification; however, it shows why these compounds have become one of the most widely used classes of drugs in clinical practice, since they are able to affect all the players involved in the immune response.

1.5 The use of glucocorticoids as anti-inflammatory drugs

The first GC introduced in clinical practice was cortisone which was employed for the treatment of rheumatoid arthritis in 1948 by Dr Philip Hench (McDonough, Curtis et al. 2008; Kadmiel and Cidlowski 2013), revolutionising the medical field. Since then numerous derivatives with GC activity have been synthesized and employed. A few examples are: hydrocortisone (cortisol, Murray 1952), prednisolone (Nobile 1958), prednisone (Oliveto 1959), methylprednisolone (Sebek 1959), dexamethasone (Muller 1961), betamethasone (Taub 1962), budesonide (Brattsand 1975) and fluticasone (Philips 1982). The clinical potency of these different GCs depends on factors such as the rate of absorption, the concentration in target tissues, the rate at which they are metabolised and cleared, and also the affinity with which they bind the main target receptor, GR. To mention just some of the differences between the most commonly prescribed steroids, hydrocortisone (HC) has a lower GR activity and higher MR affinity compared with prednisolone, while dexamethasone possesses a particularly high GR activity and a lower MR activity. These differences impact on the use of the various drugs, with HC used mostly topically, for short periods of time to avoid off-target side effects. Regardless of their clinical differences, GCs are the standard therapy for a myriad of inflammatory conditions such asthma, allergies, autoimmune diseases, dermatological condition

(dermatitis, psoriasis) and inflammatory bowel disease. This represents a very limited list though as their use has been reported in approximately 200 conditions (Zetterstrom 2008), and the number of prescriptions for these drugs has been increasing over the years; in the United States alone about 10 million of them are written for oral GCs each year, with the total annual market worth around 10 billion US dollars (Schacke, Docke et al. 2002).

Despite the widespread use of these drugs, they are strongly associated with a myriad of adverse side effects, especially in the case of long-term and/or high-dose therapy regimes. It is estimated that long-term therapy with GCs represents between 1% and 3% of the adult population worldwide, with 20% of patients using the medication for more than 6 months, and 5% taking daily GCs for more than 5 years (van Staa, Leufkens et al. 2000). In the UK, a third of the patients taking GCs for more than 2 years receive a high dose, defined as more than 7.5 mg of prednisone daily (Wei, MacDonald et al. 2004; McDonough, Curtis et al. 2008). Among the most common and troublesome side effects are osteoporosis, glaucoma, weight gain, muscle atrophy, diabetes and impairment of wound healing (Bungard, Hartman et al. 2011). A large population study identified weight gain as the side effect with the greatest prevalence, followed by skin bruising or/and thinning and sleep disorders (Angeli, Guglielmi et al. 2006). Another study conducted in France found that 71% of patients undergoing systemic therapy reported the onset of side effects in the first three months of therapy, with 46.2% of them experiencing skin abnormalities (Fardet, Flahault et al. 2007). In the US alone it is estimated that 14% of people take GCs for skin or musculoskeletal conditions (Steinbuch, Youket et al. 2004). Factors determining the occurrence and severity of the side effects are duration, dosage, potency of the GC administered and individual susceptibility (Schacke, Docke et al. 2002).

Previous *in vivo* data from my supervisors' group demonstrated that the 5α-reduced GC, 5α-THB, is an effective anti-inflammatory compound in a murine model of dermatitis. For this reason, an overview of the topical treatments with GCs and their side effects is given below.

1.5.1 Topical glucocorticoids and their side effects

GCs are the most widely used therapy employed to treat dermatological conditions (Schacke, Docke et al. 2002). They were used topically for the first time in 1952, when HC was employed to treat dermatoses (Sulzberger and Witten 1952); soon after, in 1955, the first adverse side effects caused by topical GCs were reported (Fitzpatrick, Griswold et al. 1955). Despite the fact that topical application of steroids is a safer route of delivering steroids, this route is not devoid of side effects. When they are administered topically on the skin only 1% of the applied amount is absorbed and is therapeutically effective, while 99% is removed from the skin by actions such as rubbing, washing and exfoliation (Robertson and Maibach 1982). Despite this, the small absorbed amount is sufficient to cause systemic side effects, while local ones can also be triggered by the transitory presence of local GCs (Hengge, Ruzicka et al. 2006). Among them the most common is skin atrophy, which is seen with every topical GC administered (Steinberg, Hudson et al. 2006; Franklin, Lunt et al. 2007). This phenomenon is characterised by an increase in skin transparency and the appearance of striae (Doran, Crowson et al. 2002; Lee, Oran et al. 2006). The severity of atrophy depends on different factors, among which is the potency of steroids and age, with infants and children being the most at risk due to their delicate skin. The mechanisms responsible for this side effect are suppression of cell proliferation, but also inhibition of collagen synthesis. Delayed wound healing is another serious side effect of topical steroidal therapy and is attributed to deregulation of different physiological processes, such as keratinocyte proliferation, fibroblastic activity and angiogenesis, impairment of which causes delayed formation of granulation tissue (Schacke, Docke et al. 2002; Hengge, Ruzicka et al. 2006; McDonough, Curtis et al. 2008).

The prevalence of skin diseases in the UK, and the widespread use of topical steroids for their treatment, makes the frequency of the side effects a matter of concern. According to the National Eczema Society (www.eczema.org), in the UK alone 5 million people suffer from some form of eczema (also known as dermatitis), with one in five children, and one in twelve adults affected. As a consequence, the search for new more selective compounds is relevant to topical conditions and their

treatments. Ideally, a better topical compound for skin diseases would lack the ability to cause systemic side effects should any be absorbed, and not cause local side-effects such as skin thinning and impairment of wound healing. The unpublished *in vivo* experiments mentioned earlier, and conducted by my supervisors, showed that treatment with 5α-THB did not affect skin thickness. The next step would be to study its effects on wound healing. Since one of the processes involved in the repair of injured skin is the formation of new vessels, this thesis will investigate the effects of 5α-THB on angiogenesis. As a consequence, an overview of what angiogenesis is and how GCs affect it is provided below.

1.6 Angiogenesis and glucocorticoids

1.6.1 Angiogenesis

Historically, angiogenesis has been defined as the multi-step process by which new vessels are formed from pre-existing ones (Moura, Lima et al. 2011), and it mainly refers to the post-natal remodelling of the vasculature; the formation of vessels during embryogenesis is instead referred to as vasculogenesis, and involves *in situ* differentiation of undifferentiated precursors originating from the bone marrow or residing in the wall of already formed vessels (Carmeliet and Collen 2000; Welti, Loges et al. 2013). Vasculogenesis can also take place in adults, even though the process does not give rise to a fully functional vasculature (Carmeliet and Collen 2000). The formation of new vessels is a highly regulated phenomenon that, during post-natal life, occurs in healthy individuals during wound healing, endometrial regeneration in women, muscle growth and adipose tissue espansion; while conditions such as cancer growth, diabetic retinopathy, psoriasis and rheumatoid arthritis are considered states in which pathological, dysregulated angiogenesis occurs, lasting for months or years (Andrade, Fan et al. 1987; Hadoke, Macdonald et al. 2006).

Different types of angiogenesis have been described including sprouting angiogenesis, bridging and intussusception; however, sprouting angiogenesis is the most studied and characterized, and it will be the focus of this introduction (Carmeliet and Collen 2000). The first event characterizing angiogenesis is dilation

of pre-existing vessels, caused by the release from endothelial cells of factors such as VEGF (vascular endothelial growth factor). VEGF production causes formation of the downstream effector nitric oxide (NO), which in turn leads to increased vascular permeability; as a consequence, proteins from the blood stream are able to cross the vessel wall and reach the extracellular matrix (ECM) to form a temporary scaffold that allows the migration of endothelial cells (ECs) (Carmeliet and Collen 2000). In order for ECs to migrate through the basement membrane (BM), their intercellular connections need to loosen and the ECM needs to be partially degraded to free space. These two events are coordinated by the dynamic rearrangement of proteins forming intercellular junctions, such as the platelet endothelial cell adhesion molecule 1 (PECAM-1) and the vascular endothelial cadherin (VE-cadherin), and by the activity of proteinases, including matrix metalloproteinases (MMPs), which degrade a variety of matrix constituents. MMPs also help orchestrate the angiogenic process by activating or freeing growth factors sequestered in the ECM under resting conditions (Carmeliet and Collen 2000); their activity is tightly regulated by other enzymes such as the tissue inhibitors of matrix metalloproteinases (TIMPS), which help to prevent excessive damage to the tissue. The migration and proliferation of endothelial cells gives birth first to solid cords that subsequently acquire a lumen to give rise to proper vessels. The final steps of the process involve the recruitment of non-endothelial cells which give structural and functional support to the newly formed vessels, followed by pruning and regression of the new network. A more refined description of sprouting angiogenesis, taking into consideration detailed molecular pathways, has been published recently (Welti, Loges et al. 2013); in this review, the endothelial cells involved in the process are divided into two types, tip cells and stalk cells. The former initiate and lead the angiogenic sprout throughout the process by responding to signals such as VEGF, and by interacting with the surrounding environment in a dynamic and complex fashion; the latter are proliferative cells that allow the extension of the sprout and formation of the lumen (Welti, Loges et al. 2013). Successful angiogenesis is the result of the complex interplay of pro- and anti-angiogenic factors, and of the intervention of cells from the immune system as well (i.e. macrophages). A representative image of the process is showed in Figure 1.5.

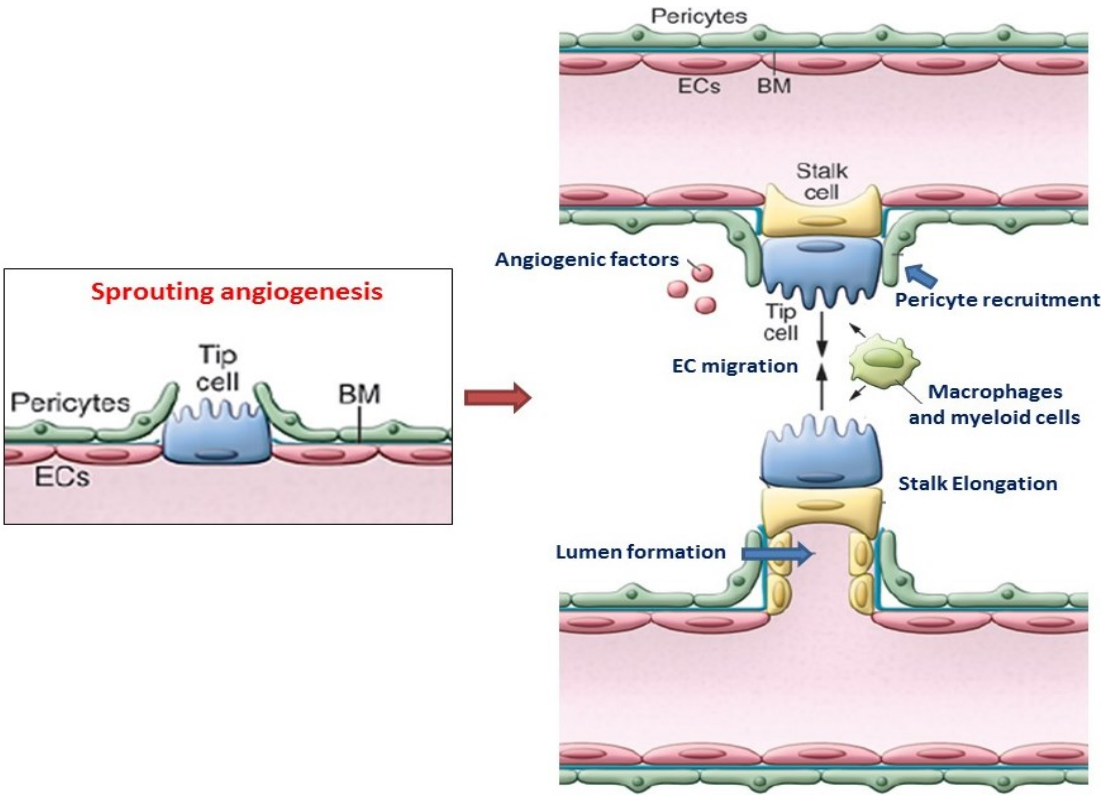

Figure 1.5 Sprouting angiogenesis. According to recent studies, two kinds of cells can be identified, tip cells and stalk cells, with the former initiating and leading the process, and the latter responsible for the elongation and formation of the tubular structure. The recruitment of peri-endothelial cells, such as pericytes, will give functional and structural stability; cells of the immune system, for example macrophages, also contribute to the process. BM = basement membrane, ECs = endothelial cells. Adapted from (Welti, Loges et al. 2013).

1.6.2 Glucocorticoids and the inhibition of angiogenesis

Cortisone and hydrocortisone, given at pharmacological concentrations (millimolar), were the first glucocorticoids to be shown to inhibit angiogenesis when given together with heparin in studies performed using the chick chorioallantoic membrane (CAM) *in vivo* assay (Folkman, Langer et al. 1983). Subsequently, other steroids were found to possess anti-angiogenic properties (Crum, Szabo et al. 1985) and, soon afterwards, an extensive investigation of a panel of steroidal molecules showed the existence of a wide group of compounds displaying the same properties; this new class of steroids was termed “angiostatic steroids” as they affected angiogenesis but were devoid of any glucocorticoid or mineralocorticoid activity. Among them was

5α-tetrahydrocortisol, which was found to be twice as potent as hydrocortisone in inducing regression of growing capillaries at pharmacological doses in the CAM assay; the compound could also inhibit, but not regress, the growth of capillaries in intracorneal tumours (Folkman and Ingber 1987). In 1996, an *in vivo* study using the subcutaneous sponge insertion model in rats revealed that dexamethasone, hydrocortisone and some of the newly discovered angiostatic steroids were inhibitors of angiogenesis without the presence of heparin (Hori, Hu et al. 1996). Soon after, it was shown that steroids were anti-angiogenic also in humans (Hasan, Tan et al. 2000). The first evidence that endogenous glucocorticoid regulation by 11β-hydroxysteroiod dehydrogenases regulates angiogenesis came from research using *ex vivo* and *in vivo* models of angiogenesis (Small, Hadoke et al. 2005; Rae, Mohamad et al. 2009) employing pathophysiological doses of the steroid.

The mechanisms by which GCs inhibit angiogenesis are not yet fully understood, and the role of GR is still controversial (Hadoke, Macdonald et al. 2006). GCs affect many of the processes taking place during angiogenesis, but the relative significance of these different influences is not yet clear. For instance, GCs inhibit the expression/activity of MMPs, and stimulate that of certain TIMPs (Perretti and Ahluwalia 2000; Pitzalis, Pipitone et al. 2002; Shikatani, Trifonova et al. 2012); furthermore, they inhibit both the expression and post-translational modifications of collagen (Kucharz 1988), and in particular they reduce the deposition of collagen IV, which is the main constituent of the basement membrane, which supports vessels during growth and maturation (Ingber, Madri et al. 1986; Maragoudakis, Sarmonika et al. 1989). As mentioned earlier, vasodilation is the initial step required for angiogenesis to begin, and it involves VEGF-induced production of NO; GCs are known to both decrease abundance of VEGF (Nauck, Roth et al. 1997; Shikatani, Trifonova et al. 2012) and inhibit the enzyme responsible for NO release, nitric oxide synthase (NOS) (Duckles and Miller 2010). In addition, some angiogenic stimuli work in concert with the inflammatory response (Leibovich, Polverini et al. 1987), and GCs are strong inhibitors of the latter. For instance, prostaglandin E2 (PGE2) and thromboxane A2 (TxA2) are factors shared by both angiogenesis and inflammation, and are also inhibited *in vitro* and *in vivo* by GCs (Herschman 1994;

Masferrer and Seibert 1994; Leahy, Koki et al. 2000; Bloomer, Kenyon et al. 2003); furthermore, MMPs are regulated by pro-inflammatory cytokines (Lohmann, Krischke et al. 2004) which are inhibited by GCs. Steroids are also known for having a negative effect on proliferation of endothelial and smooth muscle cells (Longenecker, Kilty et al. 1984; Sakamoto, Tanaka et al. 1987; Versaci, Gaspardone et al. 2002), and there are reports showing that they stimulate apoptosis of pericytes (Katychev, Wang et al. 2003). While it is still unknown which of these effects represent the major mechanism explaining why GCs inhibit angiogenesis, their broad inhibitory influences are bound to have detrimental consequences on this finely regulated process, with negative physiological repercussions; for instance, when they are used topically for the treatment of inflammatory skin conditions, at high doses and/or for a long time, they cause impairment of wound healing, a process that relies on angiogenesis. For these reason, an alternative anti-inflammatory compound that does not inhibit angiogenesis, or inhibit it to a lesser extent than the currently prescribed GCs, would be preferable.

1.7 Selective agonists of the glucocorticoid receptor

Because GCs used in clinics have an unfavourable risk/benefit ratio, over the years clinicians and researchers have tried different approaches in order to reduce the risk of adverse effects; some of these approaches have included lowering the dose, shortening the length of the treatment (e.g. several shorter periods in place of longer ones) and using GCs in combination with other drugs. However, the first two ways are not ideal because of the reduction in the effectiveness of the therapy; the last approach has been developed during the past decade with some encouraging results, for example in the treatment of rheumatoid arthritis (Zimmermann, Avery et al. 2009). Despite these newer interventions though, the search for safer compounds continues, and most of it has focussed on finding molecules using approaches based on the way in which GCs work, as outlined below.

In spite of the recognition of several mechanisms of action of the GR-GCs complex, anti-inflammatory actions of GCs are still widely thought to be mediated by DNA binding- and GR dimerisation-independent trans-repression mechanisms. This

predominant view took hold when a knock-in mouse carrying a dimerisation-deficient version of GR (called GR^{dim}) was created. This mouse model has a mutation (A458T) affecting the dimerisation domain contained in the DBD, thus preventing binding of GR dimers to the GREs of GC-responsive target genes. When GR^{dim} mice were injected with dexamethasone, GR failed to activate GRE-dependent genes *in vivo* in the liver, such as *Tat* and *Pepck*, but trans-repressed AP-1-dependent genes, such as collagenase 3 and gelatinase B, and NF-κB-dependent transcription (Reichardt, Kaestner et al. 1998; Tuckermann, Reichardt et al. 1999). These and other observations have led to the hypothesis that the side effects and anti-inflammatory properties of GCs can be uncoupled from one another, and so-called "dissociated compounds" retaining the anti-inflammatory profile but lacking the capacity to prompt adverse effects, could be generated. However, the fact that GCs can lead to expression of anti-inflammatory genes by trans-activation makes it unclear to what extent anti-inflammatory effects are independent from gene induction or to what extent therapeutic properties can be dissociated from deleterious effects (Newton 2000; Rogatsky, Wang et al. 2003; Schacke, Rehwinkel et al. 2005; Abraham, Lawrence et al. 2006). Recently, for example, it has been shown that expression of some prednisolone-induced genes was still present in the GR^{dim} mouse (Frijters, Fleuren et al. 2010), while another group reported the existence of dimerisation-independent loss of osteoblast differentiation, accounting for at least a part of GC-induced osteoporosis (Rauch, Seitz et al. 2010). In addition, the metabolic gene phenylethanolamine N-methyltransferase (*Pnmt*) has been found to be more strongly induced by GR^{dim} than by wild-type GR (Adams, Meijer et al. 2003). In this case, the mutant GR formed multimeric complexes with other regulatory factors and proteins. Nevertheless, many studies have been performed in order to identify steroidal and non-steroidal molecules with such dissociated characteristics.

These alternative anti-inflammatory compounds are, among other things, called "selective glucocorticoid receptor modulators", or SGRMs, to point out that these drugs are supposed to work selectively by inhibiting inflammatory processes while not influencing other unrelated physiological functions, as opposed to the indiscriminate behaviour of classical GCs. At present, prescriptions of synthetic GCs,

such as dexamethasone, betamethasone, triamcinolone, prednisone, prednisolone and methylprednisolone, are estimated to number about 40 million just in the UK, highlighting the vast proportion of the population in danger of suffering the powerful GCs-induced side effects, and the importance of the quest for alternative drugs. These molecules have been identified historically by drug library screenings, and tested *in vitro* for capacity to bind GR and trans-activation and trans-repression properties. Trans-activation is studied by investigating the capacity of these compounds to induce transcription of GRE-containing genes, such as *Tat*, or luciferase reporters, such as MMTV-Luc and PNMT-Luc. Trans-repression instead is investigated by studying NF-κB/AP1-dependent expression of constructs after stimulation with inflammatory stimuli. In addition, some screening for dissociated GR ligands has included the study of the inability to induce S211 phosphorylation as a read-out of their dissociated properties (De Bosscher, Vanden Berghe et al. 2005). The characteristics of the drugs under investigation are compared to those of dexamethasone, B or prednisolone, and if they are promising, they are further studied *in vitro* and *in vivo* for anti-inflammatory and side effects.

Here, I will review some of the compounds that have emerged from such a quest; Figure 1.6 gives a representation of the structures of the SGRMs described below, including also that of 5α-THB, its precursor B, and prednisolone.

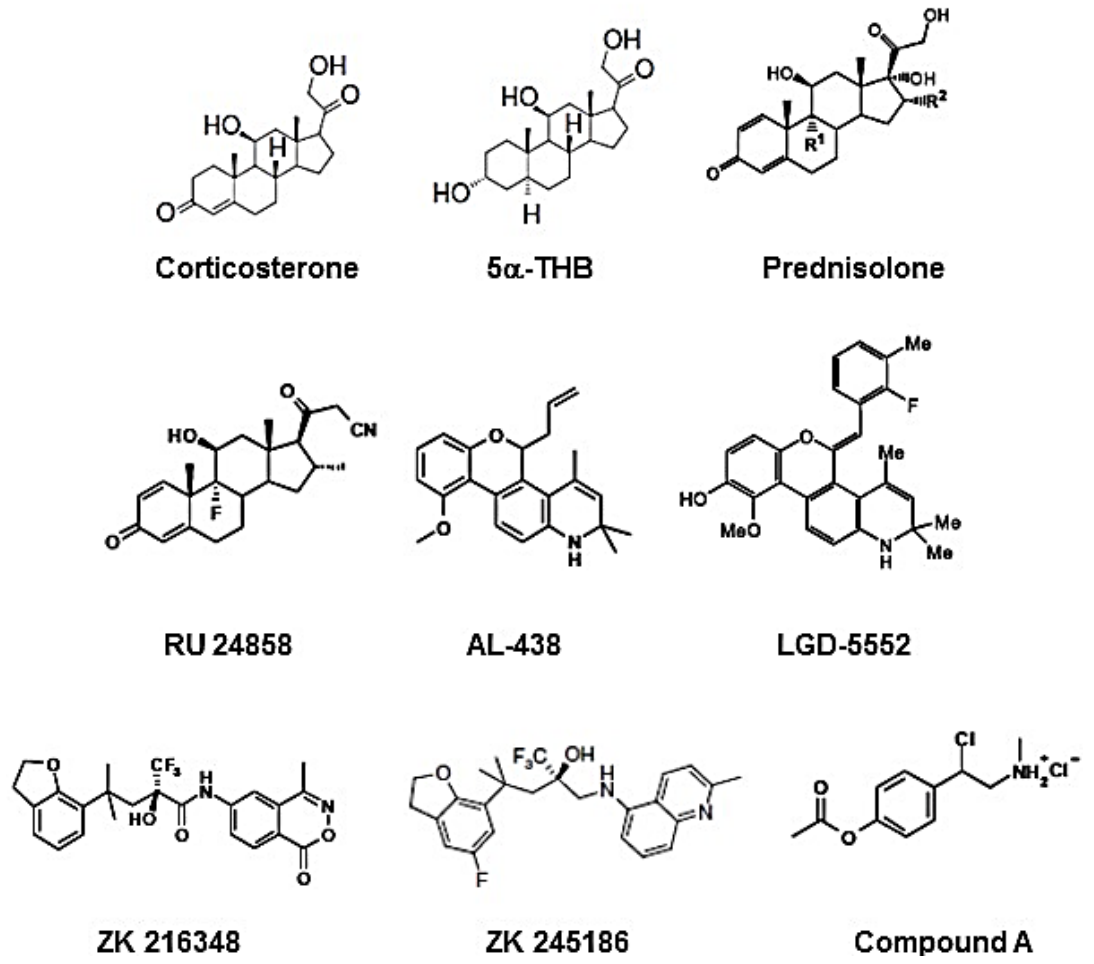

Figure 1.6 Molecular structures of selective glucocorticoid receptor modulators (SGRMs). The structures of the naturally occurring glucocorticoid corticosterone, its 5α-reduced metabolite, 5α-tetrahydrocorticosterone (5α-THB), and the synthetic glucocorticoid prednisolone, are included for comparison.

1.7.1 RU24858

This compound has a steroidal structure and was one of the first dissociated GR-ligands discovered (Vayssiere, Dupont et al. 1997; Chivers, Gong et al. 2006). Its trans-repression properties *in vitro* were similar to those of dexamethasone, while its trans-activation activity was weaker. However, *in vivo* it did not show an improved effects/side effects profile (therapeutic index) (Belvisi, Wicks et al. 2001). This molecule was useful though in the discovery of new genes with a potential role in the anti-inflammatory effects of GCs, as it was shown that it could trans-activate genes such as glucocorticoid-induce leucine zipper (*Gilz*), dual specific phosphatase 1

(*Dusp1*), and annexin 1 to the same extent as dexamethasone (Janka-Junttila, Moilanen et al. 2006).

1.7.2 AL-438

The compound Abbott-Ligand 438 (AL-438) does not have a steroidal structure, and it has been identified analysing a range of molecules for their affinity to GR, their ability to induce a reporter containing the MMTV promoter (multiple GRE sites) and to repress an E-selectin construct (containing AP-1 and NF-κB binding sites). AL-438 had a similar affinity for GR to prednisolone, a weak activity on MMTV-Luc, but strongly activates the E-selectin reporter. In addition, AL-438 was not able to inhibit the expression of osteocalcin, a marker of bone formation, as opposed to prednisolone (Coghlan, Jacobson et al. 2003). This drug was also shown to have an improved effects/side effects profile in an *in vivo* rat model of acute inflammation, having reduced side effects on glucose and bone metabolism (Humphrey, Williams et al. 2006), however these are present. Interestingly, it was shown that AL-438 promotes recruitment of different cofactors compared to prednisolone, indicating a possible explanation for its dissociated behaviour (Owens and Keyse 2007).

1.7.3 LGD-5552

LGD-5552 was recently discovered, and it is also a non-steroidal drug. After encouraging results from *in vitro* tests, it was studied in *in vivo* rat models of rheumatoid arthritis and multiple sclerosis, showing potent anti-inflammatory effects, the capacity for inducing the expression of the anti-inflammatory cytokine IL-10 and fewer side effects than prednisolone (Lopez, Ardecky et al. 2008).

1.7.4 ZK 216348 and ZK 245186

ZK 216348 and ZK 245186 were reported by Bayer Schering Pharma, and they are also non-steroidal drugs. The former was shown to be 300 times less potent than dexamethasone at inducing *Tat* in liver cells, and being able, on the other hand, to repress LPS-induced IL-8 expression in a monocyte cell line, but with lower potency and efficacy than dexamethasone and prednisolone. In *in vivo* models of skin inflammation in mice, ZK 216348 had comparable anti-inflammatory effects to prednisolone, but fewer side effects on skin and glucose levels, showing it to be a

potentially promising molecule (Schacke, Schottelius et al. 2004). The other drug, ZK 245186, after encouraging results in *in vitro* tests against MMTV promoter and AP1-dependent transcription, was tested *in vivo* in rodent models of irritant and allergic contact dermatitis, giving similar positive outcomes. However, the compound still caused skin thinning in rats after long-term (19 days) topical application to a similar extent to medium-potency GCs routinely used in clinical practice (Schacke, Zollner et al. 2009). Other studies by Bausch & Lomb investigated the molecule in ocular inflammatory conditions, gaining promising results on human corneal epithelial cells (Cavet, Harrington et al. 2010), and showing strong anti-inflammatory effects with reduced side effects in a rabbit model of ocular disease (Shafiee, Bucolo et al. 2010). After further studies in rabbits and monkeys, ZK 245186 is under clinical investigation as a topical agent in ocular inflammation after cataract surgery (phase III), allergic conjunctivitis (phase II), dry eye syndrome (phase II) and atopic dermatitis (phase II) (Ayroldi, Macchiarulo et al. 2014).

1.7.5 Compound A (CpdA)

One the most intensively studied SGRMs is a stable analogue of a molecule derived from a Namibian desert shrub, called compound A, or CpdA, first reported in 2005 (De Bosscher, Vanden Berghe et al. 2005). The drug showed higher affinity for GR than dexamethasone *in vitro*, and, moreover, it did not activate GRE-dependent constructs, such as MMTV, while it repressed E-selectin, IL-6 and IL-8 reporters to a similar extent to dexamethasone. In the same study as the compound displayed completely dissociated behaviour, phosphorylation of GR at ser211 was also investigated since it had been correlated with the transcriptional activity of the receptor. Interestingly, while dexamethasone enhanced the phosphorylation status of the residue by 10-fold over background levels, CpdA did not lead to a similar increase, correlating therefore with the lack of induction of GR-mediated transcription. Further *in vivo* studies were performed by the same group, using a murine zymosan-induced inflamed paw model. Decreased paw swelling was used as a read-out for the anti-inflammatory properties of the drug, and CpdA was shown to be as effective as dexamethasone. Analysis of the side effects revealed that CpdA did not affect blood sugar levels. Also in other *in vivo* models this compound has been

shown to be an effective anti-inflammatory drug, such as in collagen-induced arthritis and multiple sclerosis murine models. In these latter studies CpdA-treated animals were shown to have a reduced hyperglycaemia/hyperinsulinaemia, HPA axis suppression and a more positive bone marker profile (Rauner, Goettsch et al.; Dewint, Gossye et al. 2008; Gossye, Elewaut et al. 2009; Wust, Tischner et al. 2009; Rauner, Goettsch et al. 2010; van Loo, Sze et al. 2010). More recently, an investigation demonstrated that CpdA inhibited GR dimerisation, giving a potential explanation for its dissociated behaviour (Robertson, Allie-Reid et al.). Some observations though are not as positive as these results would suggest. Some authors have indeed reported that the drug causes toxic to lethal effects *in vivo*, due to its conversion into pro-apoptotic agents (Wust, Tischner et al. 2009). Moreover, it is worth noting that in all studies, the dose of CpdA used to obtain similar effects to dexamethasone was 5 to 15 times higher. This highlights the fact that this compound may not really be the "holy grail" of the quest for alternative anti-inflammatory molecules, and demonstrates the challenges posed by this field of research.

1.7.6 5α-THB

My supervisors' group identified a potential SGRM from studies of endogenous steroid metabolites. As seen earlier in this chapter, the principal metabolic clearance of GCs takes place in the liver by steroid A-ring reductases (McInnes, Kenyon et al. 2004). These enzymes catalyse a two-step A-ring reduction, which is common to other steroid hormones. GR binds preferentially steroids with a ketone rather than a hydroxyl at C3 position (Bledsoe, Montana et al. 2002), however, my supervisors' group has shown that 5α-THB can displace dexamethasone from GR binding sites in rat hepatocytes, and induce transcription of the GRE-containing, GR-responsive promoter MMTV in transiently transfected HeLa cells (McInnes, Kenyon et al. 2004). Furthermore, when 5α-THB was given to adrenalectomised rats, it suppressed circulating adrenocorticotrophic hormone (ACTH) levels (McInnes, Kenyon et al. 2004), a response normally triggered by B to mediate negative feedback on the HPA axis to lower GC production. More recent investigation carried out by my supervisors' group, showed that 5α-THB suppressed release of pro-inflammatory proteins from bone marrow-derived macrophages stimulated by LPS (Yang, Nixon et

al. 2011). In particular, at equivalent doses, 5α-THB was about three times less effective in suppressing the release of TNFα and IL-6 than B and dexamethasone, while it was as effective as B and dexamethasone in inducing secretion of the anti-inflammatory cytokine IL-10 from unstimulated macrophages. The suppressive effects of GCs on TNFα and IL-6 are mediated through binding of GR to transcription factors such as NF-κB and AP-1 (Smoak and Cidlowski 2004), while induction of IL-10 expression is due to interactions of GR with STAT3 (Unterberger, Staples et al. 2008); the data obtained by my supervisors' group indicate that 5α-THB may exert its effects by the same mechanisms (Yang, Nixon et al. 2011). Effects of 5α-THB were also investigated *in vivo* using acute and chronic models. Acute administration of 5α-THB significantly lowered the recruitment of inflammatory cells into the peritoneum in thioglycollate-induced peritonitis, and suppressed peritoneal IL-6 levels, albeit with a lower efficacy than B (Yang, Nixon et al. 2011). Chronic infusion of 5α-THB for two weeks could suppress LPS-induced secretion of TNFα and IL-6 in the blood to a similar extent to B (Yang, Nixon et al. 2011). More importantly, despite having similar anti-inflammatory actions at equivalent doses, 5α-THB did not affect glucose metabolism, in contrast to B. The drug did not induce hepatic activity of TAT, and following chronic administration, the deleterious side effects seen with B (increased blood pressure and insulin resistance) were not seen when using 5α-THB (Yang, Nixon et al. 2011). Based on these promising results, our group decided to further investigate the molecular mechanisms underlying the dissociated behaviour of 5α-THB, and this investigation is the topic of this PhD project.

1.8 Hypotheses

Given the data my supervisors' group has collected on the properties of 5α-THB, the hypotheses of this PhD are:

- 5α-THB acts as an anti-inflammatory compound by modulating molecular pathways in a different manner than its precursor B

- 5α-THB does not inhibit angiogenesis

- 5α-THB does not trans-activate conventional GC target genes

- The effects of 5α-THB are mediated by GR

1.9 Aims

To test the hypotheses, the aims of this thesis are:

- To investigate the effects of 5α-THB in *in vivo* and *in vitro* models of inflammation, and their dependency on GR

- To analyse the effects of 5α-THB on angiogenesis, a process taken as representative of the potential side effects of the compound

- To compare the effects of 5α-THB on trans-activation of genes conventionally targeted by GCs in a GR-dependent fashion

This will allow an understanding of how this compound compares with the precursor B in terms of selectivity for inflammatory pathways.

Chapter 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

Room temperature (RT) is defined as 18-22°C; the concentration of phosphate buffer solution (PBS) is 1X unless otherwise stated. Time is abbreviated as follow: sec = second/s; min = minute/s, h = hour/s, ON = overnight, n = biological replicates, N = experimental replicates.

2.1 Cell biology

2.1.1 Materials

2.1.1.1 Outsourced reagents and chemicals

Dextran-coated charcoal, ethanol (vehicle) and dimethylsulfoxide (DMSO) were from Sigma-Aldrich (Dorset, UK). All reagents for the preparation of cell culture media were from Lonza (Berkshire, UK) unless otherwise stated. Cell culture plates and all consumables were from Fisher Scientific (Loughborough, UK), with the exception of sterile syringes, needles and cell strainers which were from BD Biosciences, Oxford, UK. The provenance of other materials is specified.

2.1.1.2 Sources of cultured cells

2.1.1.2.1 Cell lines

The two murine cell lines RAW264.7 (macrophages) and L292 (fibroblasts), and the human alveolar carcinoma cell line, A549, were from the European Collection of Cell Cultures (ECACC; distributor Sigma-Aldrich). The murine hepatoma cell line, BWTG3, was a gift from Prof. Guy Haegeman, University of Ghent (Ghent, Belgium).

2.1.1.2.2 Murine bone marrow-derived macrophages (BMDMs)

Bone marrow-derived macrophages (BMDMs) were isolated from murine tibia and femur as follows, according to a protocol revised from the literature (Weischenfeldt and Porse 2008):

Bone retrieval: the mouse (inbred strain C57BL/6, male, 8-10 week old) was sacrificed by asphyxiation with CO_2 and the lower body was sprayed with ethanol (70% v/v in milliQ water). Both femurs were dislocated from the hip joints, and skin was removed from the posterior limbs. Both femurs and tibias were detached and

cleaned of soft tissues. Isolated bones were then immersed in sterile, ice cold PBS and transferred to a laminar flow cabinet where, under sterile conditions, cells were isolated.

Isolation of bone marrow-derived cells: each end of the femur and tibia were removed using a scalpel so that the marrow inside was exposed. The bone was held using sterile forceps at one of the extremities on top of a tube and, using a syringe containing F-12 medium (5 mL) attached to a needle (22 G), the marrow was flushed out until the bone looked transparent. The suspension obtained was gently passed three times through a bigger needle (18 G) in order to break cell clumps, and then transferred to a clean tube through a cell strainer (40 μm) in order to trap bone fragments and debris. The suspension was diluted in order to obtain a concentration of 5 x 10^3 cells/mL, and cells (1 mL) were plated in 12-well cell culture plates. Cells that were precursors of macrophages were allowed to attach to the surface of the plates until the next day (day 2) when the medium was removed, wells washed with PBS and fresh medium added. Cells were left to differentiate into macrophages for 6 days before the start of any experiment. The cell culture medium (section 2.1.1.3.2) was replaced every second day.

2.1.1.3 Preparation of reagents and solutions

2.1.1.3.1 Foetal bovine serum (FBS)

Heat inactivated (HI)-FBS: serum was incubated for 30 min at 56 °C in a water bath.

Stripped HI-FBS: in order to remove steroid hormones naturally present in serum, HI-FBS (500 mL) was incubated under constant agitation (ON, 4 °C) with dextran coated-charcoal (5 g) and subsequently sterilised by filtration (0.22 μm, Merck Millipore Ltd, Cork, IRL). Aliquots (50 mL) were stored at -20°C.

2.1.1.3.2 Cell culture media

Normal-serum medium: RAW264.7, A549 and BWTG3 cell were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with a high concentration of glucose (4.5 g/L glucose), HI-FBS (10% v/v), penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM). L292 cells were

cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12) supplemented with HI-FBS (10% v/v), penicillin (100 IU/mL) and streptomycin (100 μg/mL). For BMMs, the same medium was employed with addition of L292 fibroblast-conditioned medium (15% v/v) (2.1.1.3.3).

Stripped-serum medium: the media for the different cell types were prepared as above with the exception of the addition of stripped HI-FBS instead of complete HI-FBS.

2.1.1.3.3 L292 fibroblast-conditioned medium for BMDMs

L292 cells were cultured in T75 cell culture plates until fully confluent and then left for 10 days without the medium being changed. The medium was then collected, sterilised by filtration (0.22 μm) and frozen (-20 °C) for subsequent use for the culture of BMMs.

2.1.2 Cell thawing, maintenance and freezing

With the exception of BMDMs which were freshly isolated when needed, the culture of the other cells was always instigated from a frozen aliquot stored in liquid nitrogen. Frozen cells were thawed in a water bath maintained at 37°C and then transferred to a tube containing normal-serum medium and collected as a pellet by centrifugation (1000 x *g*, 5 min, RT). The cell pellet was re-suspended in normal-serum medium and seeded in a 25 cm^3 flask. The day after, cells were washed with sterile PBS and detached from the flask by mechanical treatment in the case of RAW264.7, or by addition of trypsin (5 min, 37 °C) in the case of A549, BWTG3 and L292, re-suspended in normal-serum medium and plated on 75 cm^3 flasks. Thawed cells were maintained at 37 °C in a 5% CO_2 incubator, and passaged a maximum of 10 times until the appropriate cell density was achieved prior to the beginning of experiments. At this point, after two washes with sterile PBS, the medium was replaced with stripped-serum medium. The length of time in which the cells were maintained in this medium varied according to the cell type and experiment carried out, as specified. Cells were collected for protein extraction and analysis according to the protocols described in sections 2.3.3.1.2 and 2.3.3.2.2, or for RNA isolation as described in section 2.3.1.2. The medium was collected for

cytokines analysis as described in section 2.3.3.3.2. Cells not used for experiments were harvested, re-suspended with FBS containing DMSO (15% v/v), frozen (ON, -80 °C) and then stored in liquid nitrogen.

2.1.3 Experimental procedures

2.1.3.1 Protein knock down by RNA interference (RNAi)

2.1.3.1.1 Reagents

Materials were from Insight Biotech (Middlesex, UK).

2.1.3.1.2 Procedure

Cells were seeded (3 x 10^5) in 6-well plates and cultured overnight in antibiotic-free medium in order to reach 60-80% confluence. For transfection, two solutions per well were prepared:

- Solution A: siRNA (2-8 μL, 10 μM) either targeted to the RNA of interested or not targeted to the human genome (control) in transfection medium (100 μL).

- Solution B: siRNA transfection reagent (2-8 μL) in transfection medium (100μL).

Solution A was added to solution B, mixed gently by pipetting and incubated at RT (30-45 min). Subsequently, 0.8 mL of transfection medium was added to the tube and the mixture added to cells previously washed with transfection medium. Cells were then cultured for 24 h before medium containing 2X stripped FBS, glutamine and antibiotics was added (1 mL, section 2.1.1.3.2). After 24 h incubation, medium was replaced with fresh stripped-FBS medium, and after 24 h cells were harvested and protein abundance assessed using western blotting as described (section 2.3.3.1).

2.1.3.2 Transfection of reporter plasmids in cultured cells

2.1.3.2.1 Reagents

Luria Bertani (LB) agarose and ampicillin were from Sigma-Aldrich. Other reagents were from various sources which are specified below.

2.1.3.2.2 Amplification of plasmids

Plasmid DNA (0.5 ng) was added to *Escherichia coli* cells (HB101, 50 μL; Promega, Southampton, UK) on ice. The mixture was heat shocked (42 °C, 50 sec), incubated

on ice (3 min) before being plated and incubated (ON, 37 °C) on 1.5% w/v LB agarose (1.5% w/v in sterile PBS) with ampicillin (100 μg/mL). A single colony was then selected and grown in LB with ampicillin for 7 h with vigorous shaking. This starter culture was subsequently diluted 1/1000 in 500 mL of the same medium and grown (overnight, 37 °C). Plasmid purification was carried out using a Plasmid Maxi Kit (Qiagen, West Sussex, UK) following manufacturer's instruction. Briefly, overnight cultures were subjected to centrifugation (800 x *g*, 10 min, 4 °C) and the pellet formed re-suspended in Buffer P1 (10 mL), followed by addition of Buffer P2 (10 mL) and mixed to ensure lysis of cells. After incubation (10 min, RT) the solution was added to Buffer P3 (10 mL), mixed thoroughly and incubated (10 min, 4 °C) to precipitate plasmid DNA. The suspension was subjected to centrifugation (16000 x *g*, 30 min, 4 °C) and the supernatant added to a QIAGEN-tip, where DNA binds to the column. The QIAGEN-tip was washed twice with Buffer QC (30 mL each), before DNA was eluted with Buffer QF (15 mL). DNA was precipitated by addition of 0.7x volume of isopropanol and collected by centrifugation (15000 x *g*, 30 min, 4 °C). The pellet was washed with ethanol (70% v/v) and subjected to centrifugation (15000 x *g*, 10 min, 4 °C). The pellet obtained was air-dried and re-suspended in DNA/RNase free water. DNA was quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Northumberland, UK) with absorbance recorded at 260 and 280 nm, and deemed acceptable if the ratio 260:280 was approximately 1.7-2.1.

2.1.3.2.3 Transient transfection

Cells maintained in culture were seeded using stripped-serum medium on 6 well plates ($4x10^5$ cells/well) 24 h prior to transfection. On the day of transfection, culture medium was replaced 1 hour before transfection with antibiotic-free medium. For each well to be transfected, a transfection complex containing the following components was prepared:

- Plasmid DNA of interest (variable quantity according to the type of plasmid, control or experimental). Refer to table 6.2 in Chapter 6.

- Opti-MEM reduced Serum Medium (100 μL, Invitrogen Life Technologies, Paisley, UK).

- Lipofectamine 2000 Transfection Reagent (4 μL, Invitrogen Life Technologies).

The solution was then incubated (30-40 min, RT) before being added directly to the medium of each appropriate well, in triplicate. After 24 h, fresh antibiotic-free medium was added and experimental treatment started. At completion, proteins from cells were harvested and plasmid activity tested as described in section 2.3.3.2.

2.2 *In vivo* physiology and pathophysiology

2.2.1 Source of experimental animals

Male mice (inbred strain C57BL/6, 8-10 week old) were purchased from Harlan Laboratories (UK) when needed.

2.2.2 Maintenance

Mice from commercial sources were allowed to acclimatise for one week after arrival prior to the start of each experiment. All mice were maintained under controlled conditions of light (lights on 0700-1900hs) and temperature (18-20 °C) and allowed free access to standard chow (RMI 801002; Special Diet Services, Witham, UK) and drinking water. All experiments were performed under the guidelines of the UK Home Office and the following project licences: JR Seckl, PPL No: 60/3962; KE Chapman, PPL No: 60/7874 and PW Hadoke, PPL No: 60/4523, and personal licence: A Gastaldello, PIL No: 60/13215). Animals were culled by decapitation for experiments studying angiogenesis and by asphyxiation with CO_2 for all the others.

2.2.3 Collection of tissues and preparation of plasma

Collection of solid tissues and implants was performed at cull as specified within experimental chapters. Plasma was obtained by centrifugation (10,000 x *g*, 5 min, 4 °C) from blood collected as described in the appropriate chapters. Aliquots were stored at -80 °C until needed.

2.3 Laboratory protocols

2.3.1 RNA analysis

2.3.1.1 Materials

All reagents were from Qiagen unless otherwise stated.

2.3.1.2 Total RNA extraction from cells

Cells were washed with cold PBS, and incubated on ice with QIAzol reagent (200 μL/well, 5 min). Subsequently, cells were released by scraping and the cell lysate transferred to cold RNase free-tubes. Total RNA was extracted by using an RNeasy mini kit according to the manufacturer's instructions. Briefly, chloroform (40 μL) was added, the tube's contents were mixed by vortexing and subjected to centrifugation (12000 x *g*, 15 min, 4 °C). The upper layer containing the total RNA was removed and transferred to a tube containing ice-cold ethanol (70 μL, 70% v/v). After brief mixing, the solution was transferred to an RNeasy spin column and buffer RWI added prior to centrifugation (12000 x *g*, 15 sec, RT). The flow-through was discarded and the column washed twice with RPE buffer (12000 x *g*, 15 sec, RT). After the last wash, the tube was subjected to centrifugation in order to remove any residual buffer (16000 x *g*, 1 min, RT), and RNase-free water (30 μL) was added thereafter. To collect total RNA, the tube was subjected to a further centrifugation (12000 x *g*, 1 min, RT). The total amount of extracted RNA was quantified with a NanoDrop Spectrophotometer.

2.3.1.3 Total RNA extraction from tissues and implants

Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit and following manufacturer's instruction. Briefly, tissue was immersed in RLT buffer (500μl) and homogenised using MagNA lyser green beads and a MagNA lyser centrifuge (Roche Diagnostics, West Sussex, UK) (6000 x *g*, 40 sec followed by 6000 x *g*, 30 sec). Lysates were transferred in new tubes containing proteinase K solution (16.7 μL/mL of RNase-free water), mixed and incubated (55 °C, 10 min) before being subjected to centrifugation (10000 x *g*, 3 min, RT). The supernatant was transferred to a new tube and ethanol added (100%, 0.5 volumes); after mixing, the solution was transferred to an RNeasy Mini spin column and subjected to centrifugation (8400 x *g*, 30 sec). The

column was washed with RW1 buffer (350 µL; 8400 x *g*, 30 sec) before DNase I solution was added and samples incubated (RT, 15 min). One more wash with RW1 buffer (350 µL; 8400 x *g*, 30 sec) was followed by two washes with RPE buffer (500 µL; 8400 x *g*, 30 sec and 8400 x *g*, 2 min) and a dry centrifugation (16000 x *g*, 1 min). Total RNA was eluted by adding RNase-free water (30 µL) and subjecting the column to centrifugation (8600 x *g*, 1 min). The total amount of extracted RNA was quantified with a NanoDrop Spectrophotometer.

2.3.1.4 Evaluation of RNA quality

To evaluate RNA quality, RNA samples were analysed using an Agilent 2100 expert Bioanalyzer and Agilent RNA 6000 Nano LabChip kit (Agilent Technologies, Berkshire, UK). RNA 6000 Nano gel matrix (500 µL) was pipetted into a spin filter. The tube was subjected to centrifugation (1500 x *g*, 10 min, RT) and aliquots (65 µL) were stored at 4 °C and used within 4 weeks. Prior to the assay, each RNA sample was diluted with RNase-free water, in order to have a concentration of total RNA in the range 25-500 ng/µL, which is the quantitative working range of the Bioanalyzer. Each RNA sample was then analysed according to manufacturer's instructions. Briefly, to prepare the gel-dye mix, RNA 6000 Nano dye concentrate was allowed to equilibrate to RT for 30 min. The dye was vortexed (10 sec), subjected to a brief centrifugation and added (1 µL) to an aliquot of filtered gel. After mixing well, the solution was subjected to centrifugation (13000 x *g*, 10 min, RT) and used within 24 h. A new RNA 6000 Nano chip was placed on the priming station, the gel-dye mix (9 µL) and the RNA 6000 Nano marker (5 µL) were added to the appropriate wells. Subsequently, the ladder and the samples were loaded in the remaining wells. After mixing the chip using a IKA vortex mixer (1 min), the run was initiated within 5 min; once completed, the RNA Integrity Number (RIN) and electropherograms associated with each sample were analysed using the 2100 Expert software provided by Agilent Technologies. The samples with RIN between 7 and 10 were accepted for real-time PCR analyses.

2.3.1.5 Reverse Transcription

First strand cDNA synthesis was performed using a QuantiTect Reverse Transcription kit. Any genomic DNA was removed during a first step where total RNA (250 ng) was treated with gDNA Wipeout buffer (42 °C, 2 min). Subsequently, reverse transcription was performed in a reaction mixture containing Quantiscript Reverse Transcriptase (1 μL), Quantiscript RT buffer (4 μL) and RT Primer mix (1 μL) adjusted to a total volume of 20 μL with RNase-free water. Negative controls were prepared in parallel: one mixture was prepared as above but in the absence of Quantiscript Reverse Transcriptase in order to identify samples contaminated with any remaining genomic DNA, and one mixture was prepared in the absence of RNA to identify contamination of the Reverse Transcriptase system reagents. Reverse transcription was carried out using an Eppendorf Mastercycler Gradient (Eppendorf, Stevenage, UK) with a heated lid. Samples were incubated at 42 °C for 15 min, then at 95 °C for 3 min, finally reduced to 4 °C and stored at -20 °C until needed.

2.3.1.6 Real-time PCR

Primers were from Invitrogen Life Technologies; UPL probes, Probes Master and LightCycler water were from Roche Diagnostics.

For quantification by real-time PCR a LightCycler 480 (Roche Diagnostics) was used. Primers (Table 2.1) were designed to match intron-spanning probes within the Roche Universal Probe Library (UPL), using the online software Universal ProbeLibrary Assay Design Center (Roche: http://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId=-1). Each newly made cDNA (2 μL), previously diluted 1:20 in LightCycler water, was mixed with LightCycler 480 Probes Master (5 μL), LightCycler water (7 μL), custom designed primers (10 μM, 0.1 μL each) and the corresponding UPL probe (10 μM, 0.1 μL). In parallel, for each gene tested, serial dilutions of a starting solution were prepared by pooling together all samples under analysis (2 μL each) and used to generate a

standard curve. A negative control to assess for potential contamination of reagents was prepared by using LightCycler water instead of sample.

Samples were heated for initial denaturation (95 °C, 5 min), followed by 50 cycles of PCR amplification, consisting of denaturation (95 °C, 10 sec), annealing (60 °C, 30 sec) and elongation (72 °C, 1 sec). Once the PCR programme was complete, samples were cooled (40 °C, 30 sec). All samples were analysed in triplicate and amplification curves plotted (x axis = cycle number, y axis = fluorescence). Triplicates were deemed acceptable if the standard deviation of their crossing points (Cp) was lower or equal to 0.5 cycles. A standard curve was generated (x axis = concentration, y axis = crossing point), fitted with a straight line of best fit and deemed acceptable if reaction efficiency was between 1.7 and 2.1.

Gene symbol, full name, accession No	Primers sequence	UPL
Acta2 (actin, alpha 2, smooth muscle, aorta), NM_007392.3	ctctcttccagccatctttcat tataggtggtttcgtggatgc	58
Actβ (actin, beta), NM_007393.3	accagaggcatacagggaca ctaaggccaaccgtgaaaag	64
Anxa1 (annexin A1), NM_010730.2	ctttgccaagccatcctg tgggatgtctagtttccacca	21
Ccl2 (chemokine (C-C motif) ligand 2), NM_011333.3	catccacgtgttggctca gatcatcttgctggtgaatgagt	62
Col1a1 (collagen, type I, alpha I), NM_007742.3	acctaagggtaccgctgga tccagcttctccatctttgc	19
Col1a2 (collagen, type I, alpha 2), NM_007743.2	cacctggtcctgttggaagt caccagggaagccagtca	9
Col3a1 (collagen, type III, alpha I), NM_009930.2	tcccctggaatctgtgaatc tgagtcgaattggggagaat	49
Col4a1 (collagen, type IV, alpha I), NM_009931.2	agttggaggaatgggcttg ccagggacaccctgtgag	80
Dusp1 (dual specificity phosphatase 1), NM_013642.3	tggttcaacgaggctattgac ggcaatgaacaaacactctcc	89
E-selectin (selectin, endothelial), NM_011345.2	acagcagggcaacatgaaat caactggacccattttggaa	48

Gene symbol, full name, accession No	Primers sequence	UPL
Gapdh (Glyceraldehyde 3-phosphate dehydrogenase), AY618199.1	aggcaaaagacaccgtcaag	52
	agaagatgcggctgtctctg	
Icam1 (Intercellular adhesion molecule 1), NM_010493.2	ttggagctagcggaccag	80
	ccggagctgaaaagttgtaga	
Il1β (Interleukin 1 beta), NM_008361.3	tgtaatgaaagacggcacacc	78
	tcttctttgggtattgcttgg	
Il6 (Interleukin 6), NM_031168.1	gctaccaaactggatataatcagga	6
	ccaggtagctatggtactccagaa	
Infγ (Interferon gamma), NM_008337.3	ctcaggaagcggaaaagga	60
	aaaattcaaatagtgctggcaga	
Mmp2 (matrix metallopeptidase 2), NM_008610.2	tgcagggtggtggtcatag	78
	tcacgctcttgagactttgg	
Mmp9 (matrix metallopeptidase 9), NM_013599.2	cagaggtaacccacgtcagc	7
	gggatccaccttctgagactt	
Mmp10 (matrix metallopeptidase 10), NM_019471.2	gagtctggctcatgcctacc	81
	caggaataagttggtccctga	
Nos3 (nitric oxide synthase 3, endothelial cell), NM_008713.4	tctaccgggacgaggtactg	3
	aggtcttgcacgtaggtcttg	

Gene symbol, full name, accession No	Primers sequence	UPL
Nr3c1 (glucocorticoid receptor), DQ504162.1	tgacgtgtggaagctgtaaagt catttcttccagcacaaaggt	56
Nr3c2 (mineralocorticoid receptor), NM_001083906.1	caaaagagccgtggaagg tttctccgaatcttatcaataatgc	11
Pdgfrb (platelet-derived growth factor receptor, beta polypeptide), NM_001146268.1	tgatgaaggtctcccagagg ctgcttgctgtggctcttct	1
Pecam1 (platelet/endothelial cell adhesion molecule1), NM_008816.2	cggtgttcagcgagatcc actcgacaggatggaaatcac	45
Srd5a1 (steroid 5 alpha-reductase 1), NM_175283.3	gagcgaggcagcatcatc tcagcttatggaagacaacagc	56
Tbp (TATA-binding protein), NM_013684.3	gggagaatcatggaccagaa gatgggaattccaggagtca	97
Thbs1 (thrombospondin 1), NM_011580.3	ccccaaccttcccaactc gggttgtaatggaatggacag	4
Thbs2 (thrombospondin 2), NM_011581.3	caagagaagctgccctattga atcagggaagctgttgcact	1
Timp2 (tissue inhibitor of metalloproteinase 2), NM_011594.3	aggtaccagatgggctgtga gtccatccagaggcactcat	52

Gene symbol, full name, accession No	Primers sequence	UPL
Tnfα (tumour necrosis factor alpha), NM_013693.2	ttgagatccatgccgttg	25
	ctgtagcccacgtcgtagc	
Ve-cadherin (vascular-endothelial cadherin), NM_009868.4	tcaccttctgtgaggagatgg	6
	gatgatcagcaaggtaatcactgt	
Vcam1 (vascular cell adhesion molecule 1), NM_011693.3	tcttacctgtgcgctgtgac	47
	gacctccacctgggttctct	
Vegfα (vascular endothelial growth factor alpha), NM_001287056.1	aaaaacgaaagcgcaagaaa	1
	tttctccgctctgaacaagg	
Vegfr2 (vascular endothelial growth factor receptor, 2), EU884114.1	accagagaccctcgttttca	22
	catttgcttgcaggaggttt	

Table 2.1 Details of primers and probes for real-time PCR analysis of murine genes using Roche Universal Probe Library (UPL). The reverse primer (3' → 5') for each gene is above the separation line, the forward primer (5' → 3') is below.

2.3.2 DNA analysis

2.3.2.1 Materials

All materials for DNA extraction were components of a DNeasy® Blood and Tissue Kit from Qiagen. For PCR assay a Qiagen Multiplex PCR kit was used. Materials for agarose gel electrophoresis were from Sigma-Aldrich. The DNA ladder (1500-100 bp) was from Promega (Southampton, UK).

2.3.2.2 DNA extraction

Samples were immersed in ATL buffer (180 μL) added with Proteinase K (20 μL) and incubated (56 °C, ON). To the mixture, buffer AL (200 μL) mixed with ethanol

(200 μL) was added and the solution was mixed thoroughly. The mixture was transferred into DNeasy Mini spin column and subjected to centrifugation (≥ 6000 x *g*, 1 min). The flow-through was discarded and the column washed with Buffer AW1 (500 μL) before being subjected to centrifugation (≥ 6000 x *g*, 1 min). The column was washed with buffer AW2, subjected to centrifugation (20000 x g, 3 min) before the DNA was eluted into a 1.5 ml tube by adding Buffer AE (200 mL) followed by a brief incubation (RT, 1 min) and centrifugation (≥ 6000 x *g*, 1 min). DNA was stored at -20 °C until PCR reactions were performed.

2.3.2.3 PCR analysis

Each sample (1 μL) was added to a reaction mixture containing the following: mastermix (10 μL), Sol^{n} Q (2 μL), primer mix (2 pmol/μL, 2 μL) and Qiagen water (5 μL). A negative control was included by adding Qiagen water to the mixture in place of any sample. DNA amplification was performed using a G-storm thermal cycler (G-Storm Ltd, Somerset, UK). At completion, the samples were removed and agarose gel electrophoresis performed.

2.3.2.4 Agarose gel electrophoresis

2.3.2.4.1 Solutions

TBE (5X) solution: Tris-HCl 1.1 M, boric acid 900 mM, EDTA 25 mM (pH 8) in milliQ water; TBE 0.5X solution (1 L): 100 mL TBE 5X + 900 mL milliQ water.

2.3.2.4.2 Procedure

Samples were added with loading dye (5 μL; 4 mg/mL cresol red in 40% w/v sucrose) and separated (80V, 4 h) on an agarose gel (2% w/v in TBE 0.5X). The gel was imaged using a UVITEC transilluminator and the UVIpro software (both UVITEC, Cambridge, UK).

2.3.3 Protein analysis

2.3.3.1 Western blotting

2.3.3.1.1 Materials and buffers

All reagents and chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Lysis Buffer: 25 mM HEPES, 68.5 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM NaF, 1 mM EDTA, 1% NP40, 10% glycerol, Halt protease and phosphatase inhibitor cocktail (200 μL, Thermo scientific, Northumberland, UK), one complete mini Protease inhibitor tablet (Roche Diagnostics).

Running Buffer 1X: 1 M Tris Base, 0.035 M SDS, 1 M HEPES in distilled water.

Transfer Buffer 1X: 0.025 M Tris Base, 0.2 M glycine, 10% methanol in distilled water.

TBST: Tris-buffered saline (TBS) prepared with 50 mM Tris-HCl and 150 mM NaCl plus Tween-20 0.1% v/v, in distilled water

2.3.3.1.2 Protein extraction

Each well was washed with cold PBS and incubated (4 °C, 15 min) with cold lysis buffer (100 μL). Cells were scraped and the lysate incubated with constant shaking (4 °C, 30 min) before being subjected to centrifugation (13000 x *g*, 20 min, 4 °C). The supernatant was utilised for protein quantification.

2.3.3.1.3 Protein quantification

Protein quantification was carried out in duplicate using a Bio-Rad Dc Protein Assay kit (Bio-Rad Laboratories, Hertfordshire, UK). A working reagent solution was prepared by adding reagent S (20 μL) to reagent A (1 mL). A protein standard was prepared by making serial dilutions of a bovine serum albumin (BSA, fraction V) solution (1 mg/mL) using the same lysis buffer used for protein extraction. Samples and standards (5 μL) were pipetted into wells of a 96-well plate microplate. The working reagent solution was added to each well (25 μL) prior to addition of reagent B (200 μL). The microplate was gently agitated to mix the reagents, incubated (15 min, RT) and absorbance at 750nm was recorded using a "OPTImax" microplate reader operated by SoftMax Pro4.8 software (Molecular Devices Limited, Berkshire, UK). A regression line of best fit (x axis concentration, y axis absorbance) was deemed acceptable if $r^2 > 0.98$ and the protein concentration of samples calculated. Data were deemed acceptable if duplicate differed from the mean by < 10%.

2.3.3.1.4 Gel electrophoresis and protein detection

Protein lysates (30 μg) were added to loading buffer (10:1, NuPage LDS sample loading dye:NuPAGE sample reducing agent; Invitrogen). Samples were heated (99 °C, 5 min) prior to separation by gel electrophoresis on a 12% w/v Pierce Precise protein gel (Thermo Fisher Scientific, Northumberland, UK) using the XCell Surelock Mini-Cell apparatus (Invitrogen). The samples were then transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK; 2 h, 70V) using Mini Trans-Blot Electrophoretic Transfer Cell apparatus (Bio-Rad Laboratories). After transfer, the membrane was blocked with a solution of 5% w/v milk (Bio-Rad Laboratories) in TBST for one hour (RT). Primary antibody diluted in a BSA solution (5% w/v in TBST) was then added and incubated (ON, 4 °C). Thereafter, the membrane was washed three times with TBST and incubated with the secondary antibody (60 min, RT) diluted 1:10000 in a milk solution (5% w/v in TBST). After three more washes, the blot was developed using the 800 nm channel of the Odyssey® Imaging Systems (LI-COR Biosciences, Cambridgeshire, UK), and the intensity of fluorescence of the bands quantified.

2.3.3.2 Luciferase and β-galactosidase assays

2.3.3.2.1 Materials

Reagents were from Sigma-Aldrich.

2.3.3.2.2 Preparation of cell lysate

Cells were washed with cold PBS and incubated (4 °C, 15 min) with lysis buffer (100 μL, 2 5mM Tris phosphate, 2 mM dithiothreitol (DTT), 1% Triton X100, 10% glycerol in distilled water). Cell lysates were transferred into 1.5 mL tubes and subjected to centrifugation (12000 x *g*, 1 min, 4 °C). Luciferase and β-galactosidase activities were assessed in the supernatant.

2.3.3.2.3 Luciferase Assay

Luciferase activity is commonly assessed to test the degree by which a promoter is induced. The enzyme luciferase, the expression of which is under the control of the promoter being investigated, catalyses the reduction of luciferin to oxyluciferin in the presence of ATP Mg^{2+} producing light as a by-product. This reaction is energetically

very efficient therefore allowing, by recording the amount of light produced, a robust estimation of the relative amount of promoter activity compared to a control.

Luciferase assays were carried out on 96-well plates with samples analysed in duplicate. To each well the following components were added: assay buffer (100 μL, 40 mM Tricine, 66 mM DTT, 0.2 mM EDTA, 2.14 mM $MgSO_4$, 0.26 mM Coenzyme A in distilled water), 0.1 M ATP (5 μL) and cell lysate sample (40 μL). To assess the background signal two wells were prepared by replacing the cell lysate with lysis buffer. Subsequently, a Microplate Luminometer (Orion II, Hertfordshire, UK) was used to add the substrate luciferin (105 μL; 1 mM) into each well, and the amount of chemi-luminescence was recorded at 560 nm.

2.3.3.2.4 β-Galactosidase Assay

Galacton is a substrate used for sensitive quantification of β-galactosidase activity in reporter gene assays. Light emitted from the reaction is directly proportional to the number of β-galactosidase enzyme molecules. To assess β-galactosidase activity, to each well of a 96-well plate, reaction mix (67 μL, 1/100 dilution of Galacton in diluent, Tropix Kit, Applied Biosystems) and the cell lysate sample (10 μL) were added immediately after the luciferase assay. To assess the background signal two wells were prepared by replacing the cell lysate with lysis buffer. The plate was incubated (15 min, RT) prior the addition of Accelerator II (100 μL, Tropix Kit, Applied Biosystems) using the luminometer as above (section 2.3.3.2.3).

2.3.3.2.5 Data management

Data were recorded using the software Simplicity 4.1 (Orion II, Hertfordshire, UK). Samples were analysed in duplicate and data deemed acceptable if they differed from the mean by < 10%.

2.3.3.3 Quantification of cytokines by Enzyme Linked Immuno- Sorbent Assay (ELISA)

2.3.3.3.1 Reagents

"ELISA READY-SET-GO!" kits were from eBioscience (Hatfield, UK).

2.3.3.3.2 Medium collection

Culture medium was pipetted into a tube and subjected to centrifugation (1000 x *g*, 10 min, 4 °C). Supernatant was immediately used for ELISA assay or stored at -80 °C.

2.3.3.3.3 Assay procedure

The amounts of cytokines released into the medium of cultured cells were quantified in duplicate according to the manufacturer's instructions. A 96-well plate was coated overnight at 4 °C with appropriate capture antibody (100 µL/well). The plate was then washed 5 times with wash buffer (PBS-0.05% Tween-20) before blocking with 1X Assay Diluent (200 µL/well, 1 h, RT). The plate was washed again as before, and the standards and samples added to the appropriate wells and incubated (ON, 4 °C). After 5 more washes, the detection antibody (100 µL/well) was added and incubated (1 h, RT). The washes were repeated and Avidin-HRP added (100 µL/well) and incubated (30 min, RT), followed by 7 more washes. Finally, the Substrate Solution (100 µL/well) was added and incubated (15 min, RT) followed by Stop Solution (50 µl/well). The absorbance was quantified at 450 nm using a "OPTImax" microplate reader. A 4-parameter standard curve was fitted (x axis log concentration, y axis absorbance), deemed acceptable if $r^2 > 0.98$, and the cytokine concentration of samples calculated. Data were deemed acceptable if duplicate differed from the mean by < 10%.

2.3.3.4 Quantification of corticosterone in plasma by ELISA

2.3.3.4.1 Materials

ELISA kits containing all the reagents, corticosterone concentrated standard and 96-well microplate were from ENZO Life Sciences (Exeter, UK); ethyl acetate was from Sigma-Aldrich; glass tubes and pipettes were from BD Biosciences.

2.3.3.4.2 Steroid extraction from plasma samples

Plasma was thawed from -80 °C in ice, and diluted 1:20 with sterile deionized water in glass tubes. An equal volume of steroid displacement reagent (SDR, diluted 1/100 with the assay buffer from the kit) was added and the mixture incubated (5 min, RT). Reagent AB15 (280 µL) and ethyl acetate (3 mL) were added to each sample; the

mixture was vigorously vortexed, allowed to stand (2 min) and then vortexed one more time. The upper, clear, organic layer was removed using a glass pipette and transferred to a fresh tube. Samples were reduced to dryness under oxygen-free N_2 at 60 °C, and thereafter the residues stored at -80 °C until quantification of corticosterone was performed.

2.3.3.4.3 Assay procedure

Reagents from the kit were allowed to warm up at RT for at least 30 min prior to beginning of the procedure. The samples were reconstituted with AB15 (500 µL) and vortexed vigorously. Standard curves were prepared by making serial dilutions of a concentrated solution provided in the kit (200,000 pg/mL) and using reagent AB15 as the diluent. Standards (100 µL) and samples were pipetted into the appropriate wells, before blue conjugate (50 µL) was added, followed by yellow antibody (50 µL). The microplate was then incubated on a plate shaker (RT, 2 h, 500 rpm). Thereafter, the wells were washed (x3) using wash solution (400 µL) before adding pNpp substrate (200 µL) followed by incubation at RT (1 h). Stop Solution (50 µL) was added to every well and absorbance read immediately at 405 nm using a "OPTImax" microplate reader. A 4-parameter standard curve was fitted (x axis log concentration, y axis absorbance), deemed acceptable if $r^2 > 0.98$ and the concentration of corticosterone in samples calculated. Samples were analysed in duplicate, and data were deemed acceptable if they differed from the mean by < 10%.

2.3.3.5 Quantification of myeloperoxidase (MPO) activity in tissue

2.3.3.5.1 Materials

MPO activity was quantified using a Myeloperoxidase fluorometric detection kit from ENZO Life Sciences (Exeter, UK). Additional materials needed and not included in the kit were: DMSO, hexadecyltrimethylammonium (HTA-Br) and black 96-well plates, all from Sigma-Aldrich

2.3.3.5.2 Sample preparation

Ear biopsies were placed in dry ice and weighed before homogenization. They were transferred into MagNA lyser green beads tubes containing 1X assay buffer and

homogenized using the MagNA lyser centrifuge (Roche Diagnostics) (6000 x *g*, 40 sec followed by 6000 x *g*, 30 sec). Homogenate was transferred into 1.5 mL tubes and subjected to centrifugation (1000 x *g*, 4 °C, 20 min). Supernatant was removed and the pellet re-suspended in solubilisation buffer (1 mL, 0.5% HTA-Br (w/v) in assay buffer). Samples were sonicated (30 sec) and submitted to two cycles of freeze/thaw, before being subjected to centrifugation (8000 x *g*, 4 °C, 20 min). The supernatant was assayed immediately for MPO activity.

2.3.3.5.3 Assay procedure

A standard curve was prepared immediately before the assay was performed by making serial dilution of the enzyme using the assay buffer. Samples or standards (50 μL) were pipetted to the bottom of a black 96-well plate, and Reaction Cocktail (50 μL) added to each well. The plate was incubated (RT, 45 min, in the dark) and fluorescence measured using a Magellan Infinite M1000 plate reader (Tecan UK Ltd, Reading, UK) with excitation wavelength set to 570 nm, and emission to 600 nm. A linear standard curve was fitted (x axis concentration, y axis fluorescence) by Tecan I-CONTROL I.6 software and deemed acceptable if $r^2 < 0.98$. Samples were analysed in duplicate and deemed acceptable if they differed from the mean by < 10%.

2.3.4 Histological analysis

2.3.4.1 Materials

Solutions for haematoxylin and eosin (H&E) staining were from Sigma-Aldrich.

2.3.4.2 Sample preparation

Tissue samples were fixed in formalin (10% v/v in PBS) for 24 h and then transferred to ethanol (70% v/v in milliQ water) prior to embedding in paraffin. Sections of 5 μm were cut using a microtome, floated in a water bath at 45 °C and placed on positively-charged glass slides (VWR International Ltd., Leicestershire, UK) and oven dried (37 °C, ON).

2.3.4.3 H&E staining

Paraffin was removed from samples by immersion in xylene (5 min, x2) before re-hydration was performed by immersion in consecutive solutions containing decreasing concentration of ethanol (100%, 95% and 70% v/v in milliQ water). After rinsing in tap water, samples were stained first with haematoxylin (5 min) and then with eosin (13 sec) in order to differentiate between cell nuclei and other cellular structures. Samples were de-hydrated by immersion in consecutive solutions with increasing concentration of ethanol (70%, 95% v/v in milliQ water and 100%), and finally cleared by using two changes of xylene prior to application of a thin layer of mountant (D.P.X., Sigma-Aldrich) and covered with a glass cover slip.

2.4 Data analysis

All data were analysed using GraphPad Prism6 software, and presented as mean ± SEM. Data were analysed by the statistical tests described in the legend of each figure. A comparison was considered statistically significant when $p < 0.05$. When a trend is reported, it refers to a p value between 0.05 and 0.1.

Chapter 3

ANTI-INFLAMMATORY

PROPERTIES OF 5α-THB *IN VIVO*

Chapter 3: Anti-inflammatory properties of 5α-THB *in vivo*

3.1 Introduction

Glucocorticoids (GCs) are the main topical drugs prescribed for the treatment of inflammatory skin conditions such as psoriasis and dermatitis. As with systemic administration, long-term topical application of GCs is restricted by the onset of serious side effects including skin atrophy, characterised by loss of skin thickness and elasticity, and impeded wound healing. In addition, variability in the response to the treatment and the onset of resistance to glucocorticoid therapy are two factors that sometimes make these drugs not as effective as clinically needed (McDonoughAK 2008; Schacke H 2002). It is, therefore, a priority for the scientific community to research and develop new, more selective anti-inflammatory drugs for topical use.

Preliminary unpublished data from my supervisors' laboratory showed that the 5α-reduced glucocorticoid 5α-THB, originating from the reduction of the principal active glucocorticoid in rodents, corticosterone (B), represents a promising candidate. This compound reduced inflammatory swelling associated with irritant contact dermatitis in a murine model of the condition. The effect was comparable to that of hydrocortisone (HC), which is a steroidal drug prescribed for the treatment of inflammatory skin conditions in humans. Long-term topical application of 5α-THB alone did not induce skin thinning or systemic metabolic effects, such as weight loss, nor did it increase insulin levels, even when given at a concentration eight times higher than its EC_{50} (25 μg). In contrast, HC caused weight loss and increased insulin levels and also induced massive skin thinning. These and earlier results (Yang, Nixon et al. 2011) suggested that 5α-THB is a more selective anti-inflammatory glucocorticoid. For these reasons, we proposed to determine the molecular mechanisms through which this steroid is exerting its anti-inflammatory action using the same murine model of irritant dermatitis.

The presence of the enzyme responsible for the formation of 5α-THB, 5α-reductase type 1 (5α-R1), has been shown in human and rat skin (Nixon, Upreti et al. 2011). Consequently, the response of any steroid, which is a substrate for 5α-R1 (e.g. hydrocortisone, corticosterone), may be modified by its presence. In addition, 5α-THB could be reversed to B, although the kinetics of the enzyme suggests this is unlikely (Nixon, Upreti et al. 2011). These concepts were explored in mice with genetic disruption of 5α-R1.

Hypotheses

- Resolution of inflammation by 5α-THB shares some but not all of the mechanisms of corticosterone (B)

- Local formation of 5α-THB plays a role in determining the local anti-inflammatory response to B

Objectives

To investigate:

- How the anti-inflammatory properties and mechanisms of 5α-THB compare with those of B

- Whether the action of 5α-THB is dependent on the glucocorticoid receptor (GR)

- Whether the presence of 5α-R1 modifies the anti-inflammatory effects of B

3.2 Materials and Methods

3.2.1 Model of contact irritant dermatitis

3.2.1.1 Materials

Croton oil (CO), isopropyl myristate (IM), ethanol, DMSO and RNA*later*® were from Sigma-Aldrich (Dorset, UK). B, 5α-THB and the GR antagonist, RU486, were from Steraloids (Newport, RI, USA).

3.2.1.2 Induction of dermatitis

Male C57BL/6 mice (9-12 week old) were treated on the inner surface of the right ear with a solution containing the irritant compound croton oil (10 μL, 3% v/v in a mixture of ethanol and IM 30:5), using a micropipette. The solution was prepared immediately prior to each experiment. The left ear of each mouse was left untreated and used as a control as preliminary experiments showed lack of effect of the vehicle. Treatments were performed between 10 a.m. and 3 p.m. in order to avoid the influence of circadian variation of steroid levels on the inflammatory response. Treatments lasted for a period of 6 or 24 h, as stated.

3.2.1.3 Treatment with steroids and glucocorticoid receptor antagonist

3.2.1.3.1 Topical treatments

A mixture (10 μL) of croton oil and steroids was applied to the inner surface of the right ear of each mouse, leaving the left ear untreated as a control. The mixtures were prepared as follows:

Topical treatment with B and 5α-THB: concentrated solutions (8 μg/μL) of B and 5α-THB in ethanol were stored at -80 °C. Working solutions were prepared before the beginning of each experiment by warming the stock solutions (RT, 1 h) and diluting them in the croton oil solution (3% v/v in a mixture of ethanol and IM 30:5).

The concentration of B to be used and compared with 5α-THB was determined by performing dose-response (swelling) experiments lasting 6 and 24 h. The doses of B tested were 0.3, 1, 3, 10 and 30 μg per mouse (corresponding to approximately 0.09, 0.29, 0.9, 2.9 and 9 mM). From the graphs generated from these experiments, the concentration of B able to reduce the inflammatory swelling by 50% (half maximal

effective concentration, also known as EC_{50}) was calculated at each time point by fitting a "non-linear log(agonist) versus response (three parameters)" regression curve using GraphPad 6 software which uses the following equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)))

The EC_{50}s for B (10 μg (2.90 mM), 6 h and 5 μg (1.45 mM), 24 h) so determined were employed in the subsequent experiments. 5α-THB was used at final concentrations corresponding to 1, 3 and 5 times the EC_{50} of B. In previous studies, in which the treatment with 5α-THB lasted 24 h, the EC_{50} was found to be 25 μg (7.13 mM) (Livingstone, Sykes et al. 2014); no previous studies are available in which the treatment lasted 6 h.

Topical treatment with RU486: RU486 was used at the same molar concentration corresponding to the EC_{50} of B at 24 h (5 μg = 1.45 mM). To prepare it, RU486 (10 μg/μL) was dissolved in DMSO prior to each experiment and diluted to 0.62 μg/μL in the croton oil solution (3% v/v in a mixture of ethanol and IM 30:5) before topical application of 6.20 μg (1.45 mM) on the mouse ear for 24 h.

3.2.1.3.2 Subcutaneous injection of RU486

A concentrated solution was prepared by dissolving RU486 either in DMSO or ethanol to a concentration of 25 mg/mL or 20 mg/mL, respectively. The mixture (0.5 mg/mouse) was then injected subcutaneously in the neck region 15 min prior to any topical treatment. The same amount of vehicle (DMSO or ethanol) was injected in a separate group of mice serving as control.

3.2.1.4 Tissue collection

At the end of each experiment, ears were collected at cull (section 2.2.2) and halved; each half was either preserved in 10% v/v formalin for histological analysis (section 2.3.4), in dry ice or RNA*later*® for real-time PCR analysis (sections 2.3.1.5 and 2.3.1.6) or frozen in liquid nitrogen for myeloperoxidase activity assay (section 2.3.3.5).

3.2.1.5 Assessment of inflammatory swelling

Inflammatory swelling (or oedema) of auricular tissues was evaluated by measuring the weight difference between treated (right) and untreated (left) ears (referred to also as control ears).

3.2.1.6 Microscopic quantification of swelling and cell infiltration

Pictures of ear tissues stained with H&E and magnified 100X under a microscope were captured using the software QCapture Pro 7 (QImaging, Canada). Quantification of swelling was performed by measuring the width (in μm) of the dermis at both sides of the central cartilage layer. Total width was calculated by summing the two measurements for each section. Quantification of cell infiltration was performed by counting the nuclei on both sides of the cartilage and summing the numbers. In order to make measurements robust, for each image the widest part and the area most populated by inflammatory cells were always analysed. Help for these analyses was received from MSc student Amber Abernethie and Honour student Nicola Tsang. The person performing the measurements was blind to group allocations.

3.2.1.7 Quantification of transcripts by real-time analysis

3.2.1.7.1 Optimisation of the method for collecting and preserving auricular tissues

Two methods for collecting and preserving ears were tested: (a) storage in dry ice upon collection and subsequent preservation at -80 ºC, and (b) storage in RNA*later*® upon collection, followed by storage at 4 ºC ON and subsequent preservation at -20 ºC. In order to evaluate the best approach, extraction of total RNA, RNA quality evaluation using an Agilent 2100 expert Bioanalyzer and Agilent RNA 6000 Nano LabChip kit, and real-time PCR analysis for the housekeeping genes TATA-binding protein (*Tbp*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were carried out as described in section 2.3.1.

3.2.1.7.2 RNA isolation

Total RNA was isolated from sponges as described in section 2.3.1.3.

3.2.1.7.3 Real-time PCR analysis

Production of cDNA and subsequent real-time PCR analysis were performed as described in sections 2.3.1.5 and 2.3.1.6.

3.2.1.8 Model of dermatitis in adrenalectomised mice

3.2.1.8.1 Surgery

Surgery was performed by Dr Dawn Livingstone. In summary, a bilateral adrenalectomy through dorsal incisions under isoflurane anaesthesia was performed. Buprenorphine pain relief was given peri-operatively and 16 h later. After surgery, animals were maintained on 0.9% saline drinking water and allowed to recover for one week before subsequent intervention.

3.2.1.8.2 Topical treatment

Contact irritant dermatitis was induced and steroid treatment applied (24 h) as described in sections 3.2.1.2 and 3.2.1.3, one week after surgery.

3.2.1.8.3 Tissue collection and assessment of inflammatory swelling

Ears were collected at cull as described in section 3.2.1.4, and swelling assessed as described in section 3.2.1.5.

3.2.1.9 Model of contact irritant dermatitis in mice with genetic disruption of 5α-R1 (5α-R1$^{-/-}$)

3.2.1.9.1 Animals

Homozygous 5α-reductase type 1 knockout (5α-R1$^{-/-}$) mice were obtained by in-house breeding of heterozygous mice (5α-R1$^{+/-}$) derived through backcrossing for >10 generations onto a C57BL/6 background. Embryos comprising the original colony (C57BL6/SvEv/129 mixed background; (Mahendroo, Cala et al. 1996; Mahendroo, Cala et al. 1997) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA).

3.2.1.9.2 Maintenance

Mice were maintained as described in section 2.2.2.

3.2.1.9.3 Genotyping of 5α-R1$^{-/-}$ mice by PCR

Materials: Ear punches for genotyping were collected at the MVM Biological Services (University of Edinburgh) by technical staff and stored at -20 °C until DNA extraction and genotyping were performed. The sources of all reagents are described in section 2.3.2. Primers were designed by Dr Dawn Livingstone (Table 3.2) and purchased from Invitrogen Life Technologies.

Genotype	Primer sequence
5α-R1$^{-/-}$	gattgggaagacaatagcaggcatgc
5α-R1 WT	atggagttggatgagttgtgc
Common	ccagacacgaacttccacgcttctg

Table 3.1 Sequences of the primers (5´→ 3´) for the genotyping of mice with genetic disruption of 5α-reductase type 1 (5α-R1). 5α-R1$^{-/-}$ = mice homozygous for the genetic modification, WT = wild type mice.

Procedure: DNA extraction and PCR analysis were performed as described (sections 2.3.2.2, 2.3.2.3). The samples were amplified with the following program: 94°C, 15 min; (94°C, 30 sec - 60°C, 1.30 min - 72°C, 1 min) x30 cycles; 72° 10 min. Products were separated by gel electrophoresis (section 2.3.2.4). The anticipated bands for genotype distinction were as follows: 5α-R1 WT band = 400 base pairs (bp); 5α-R1$^{-/-}$ band = 360bp.

3.2.1.9.4 Topical treatment of 5α-R1$^{-/-}$ mice

Topical treatment with croton oil alone or in conjunction with steroids lasting 6 and 24 h was performed on male 5αR1$^{-/-}$ mice (9-12 week old) as described (sections 3.2.1.2, 3.2.1.3). As control, littermate wild type (5α-R1 WT) male mice were used.

3.2.1.9.5 Collections of tissues and assessment of inflammatory swelling

Ears were collected at cull as described in section 3.2.1.4, and swelling assessed as described in section 3.2.1.5.

3.2.1.9.6 Collection of blood

Blood was collected immediately after removal of the ears, in tubes treated with sterile EDTA solution (0.5M, pH 8) by puncture of the abdominal aorta using a 26G needle inserted onto a 1 ml syringe (both from BD Biosciences, Oxford, UK). Plasma was recovered as described in section 2.2.3.

3.2.1.9.7 Quantification of B in plasma by ELISA

B in plasma was quantified as described in section 2.3.3.4.

3.2.2 Analysis of 5α-reductases in murine skin by PCR

3.2.2.1 Materials

Sources of reagents are described in sections 2.3.1 and 2.3.2. Specific primers for 5α-R1 and 5α-Reductase type 2 (5α-R2) were designed by Dr Dawn Livingstone (Table 3.1) and purchased from Invitrogen Life Technologies (Paisley, UK).

Gene symbol, full name, accession No	Primers sequence	
Srd5a1 (steroid 5 alpha-reductase 1), NM_175283.3	R	ctgccatcaattccttggat
	F	tttgctcttcctttgggcta
Srd5a2 (steroid 5 alpha-reductase 2), NM_053188.2	R	cgcgcaataaaccaggtaat
	F	aacacagcgagagtgtgtcg

Table 3.2 Sequences of the primers for PCR analysis of 5α-reductases type 1 and 2. R = reverse, F = forward.

3.2.2.2 PCR analysis

Total RNA and cDNA from murine ears, liver and prostate were obtained as described in sections 2.3.1.3 and 2.3.1.5. Samples were amplified by PCR (section 2.3.2.3) using the following protocol: initial denaturation = 95 °C, 5 min; (94 °C, 4 sec – 55 °C, 45 sec – 72 °C, 1 min) x33 cycles; final extension 72 °C, 10 min. The products were separated by agarose electrophoresis (section 2.3.2.4). The anticipated bands were: 5α-R1 = 240 bp; 5α-R2 = 299 bp.

3.2.3 Data analysis

3.2.3.1 General

Data were represented as mean ± SEM, and analysed as described in section 2.4.

3.2.3.2 Analysis of the inflammatory response in ear

The effects of the application of steroids on ear swelling, cell infiltration and thickness are presented as percentage of the group that received CO alone; the mean weight difference in this group was set as 100% inflammation and efficacy of steroids on different parameters calculated accordingly. The inflammatory swelling was reported as weight difference in mg when the comparison did not involve steroidal treatment.

3.2.3.3 Real-time PCR analysis

For quantification of transcripts by real-time PCR, the abundance of each gene was represented in relation to the abundance of chosen housekeeping genes. For this chapter, the two housekeeping genes used were *Tbp* and *Gapdh* due to the lack of significant changes in the abundance of transcripts following treatment with croton oil or croton oil plus steroids. For each sample and each gene studied the values presented in the graphs were calculated as follow: value sample X/(mean values *Tbp* + *Gapdh* sample X).

3.3 Results

3.3.1 Characterization of a model of irritant dermatitis

3.3.1.1 Inflammatory swelling in response to croton oil

Application of croton oil induced tissue swelling after both 6 h and 24 h (Figure 3.1); the effect was greater at 6 h compared with 24 h.

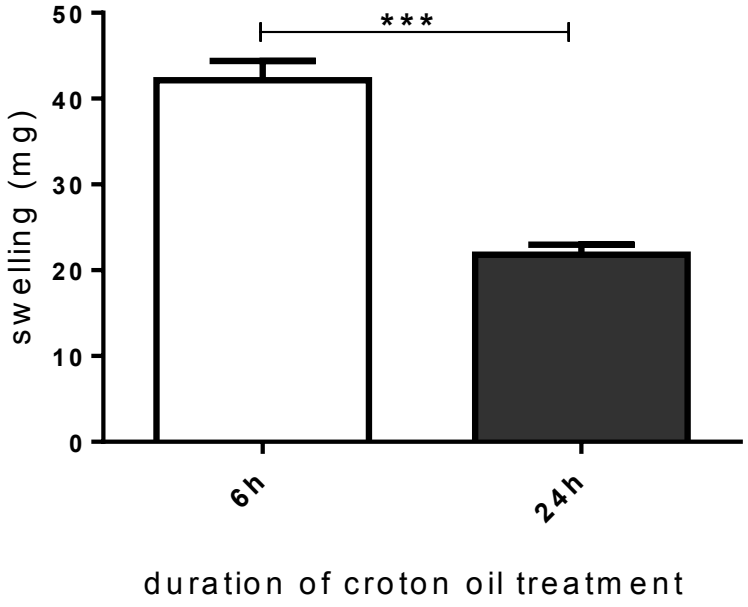

Figure 3.1 Ear swelling induced by croton oil was greater after treatment for 6 than 24 hours (h). Weight difference (mg) between treated and untreated ears after treatment for 6 and 24 h with croton oil. Data (mean ± SEM) were analysed by Student's t-test; n=10/group, *** = $p < 0.0001$.

3.3.1.2 Histological changes associated with inflammatory swelling

Staining of auricular tissues with H&E showed an increase in the dermal thickness of ears treated with croton oil for 6 and 24 h compared with the matched untreated ears; the swelling appeared greater at 6 h (Figure 3.2 (6h) A,B,C; (24h) D,E,F). This confirmed measurements of ear weights at cull (Figure 3.1). At 24 h-post treatment, generalised cell infiltration was more abundant than at 6 h, seen clearly at higher magnification (Figure 3.2 C & F).

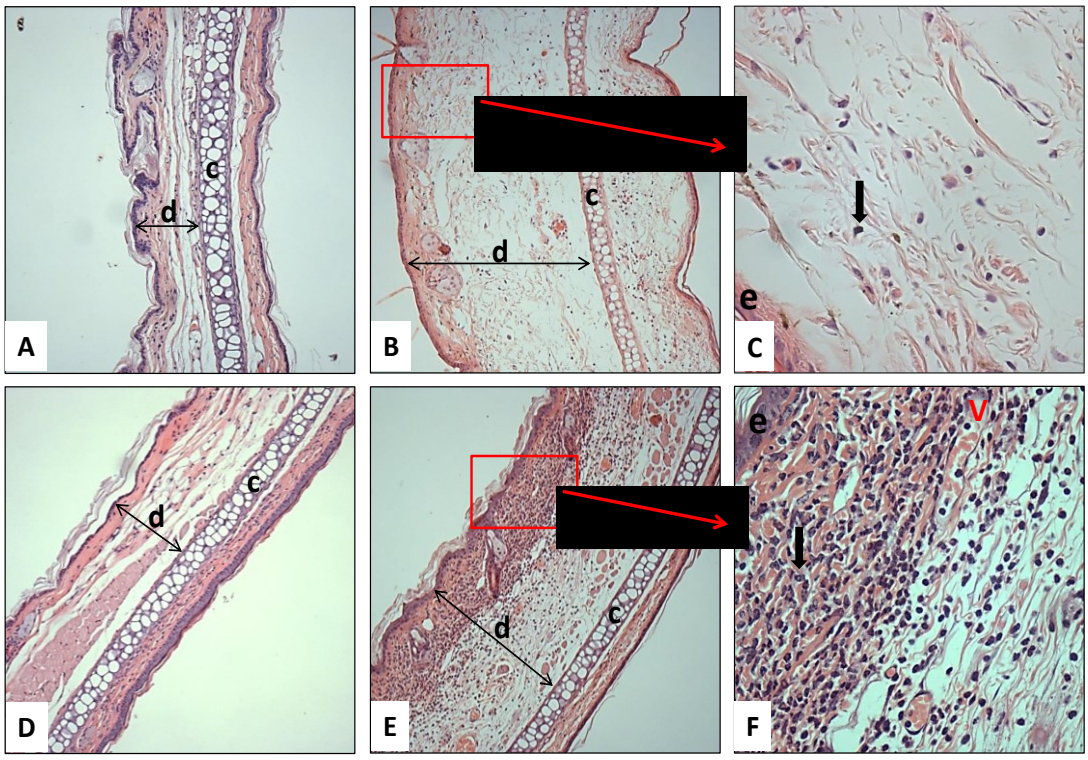

Figure 3.2 Treatment of ears with croton oil for 6 hours (h) caused a greater dermal swelling, but less cell infiltration, compared with treatment lasting 24 h. Representative images of tissues stained with haematoxylin and eosin from untreated (A, D) and croton oil-treated (B, E) murine ears. Ears were collected after treatment for either 6 h (A, B) or 24 h (D, E). Selected fields in B and E were magnified (400x) in order to show cell infiltration (C and F, black arrow indicating purple nuclei). All other images were taken using a 10x objective. c = cartilage; d = dermis, which can be found at both sides of the ear cartilage; only one dermal layer has been highlighted for clarity; e = epidermis.

3.3.1.3 Evaluation of the method to collect and preserve tissues for real-time PCR analysis

3.3.1.3.1 Collection in dry ice

The RNA quality, as evaluated by gel electrophoresis (Figure 3.3 a), was poor for samples belonging to the croton oil-treated group (samples 9-12, 6 h; 13-16, 24 h) compared with samples of the control group (samples 1-4, 6 h; 5-8, 24 h), as shown by the low RNA integrity number (RIN) and the high number of bands indicating degradation of the RNA pool. This translated into poor quality data obtained by real-time PCR analysis; indeed, the abundance of mRNAs of the housekeeping gene *Tbp* was lower in the treated group at both time points compared with the control group (Figure 3.3 b). Analysis of another housekeeping gene, *Gapdh*, showed that the amount of transcripts was not different between the treated and untreated group for both end points (Figure 3.3 c), however the treated group at 24 h showed a substantial error around the mean.

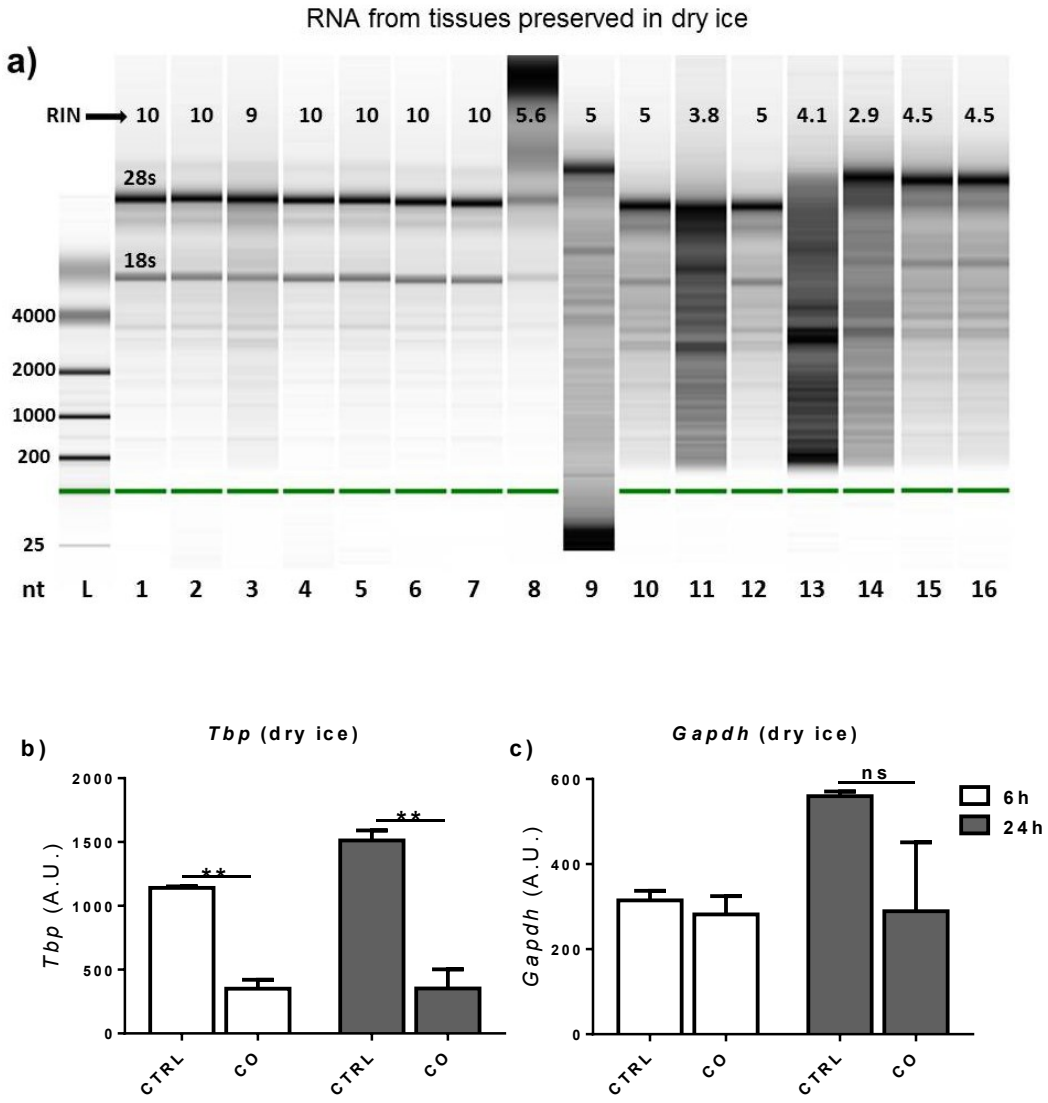

Figure 3.3 Collection of auricular tissues in dry ice yielded poor quality RNA. (a) Gel electrophoresis of either untreated (1-4, 6 hours (h); 5-8, 24 h) or croton-oil treated ears (9-12, 6 h; 13-16, 24 h) collected in dry and stored at - 80°C; nt = number of nucleotides, L = RNA ladder, RIN = RNA integrity number, 28s (5070 nt) and 18s (1869 nt) = sizes of the correspondent ribosomal RNAs in Svedberg units. (b) and (c) Abundance of transcripts of TATA-binding protein (*Tbp*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) respectively in control (CTRL) and croton (CO)-treated ears quantified through real-time PCR at 6 and 24 h. Data (mean ± SEM) were analysed at each time point by unpaired Student's t-test; ** = $p < 0.001$, ns = not significant; n = 4/group. A.U. = Arbitrary Unit.

3.3.1.3.2 Collection in RNA*later*®

The RNA quality, as evaluated by gel electrophoresis (Figure 3.4 a), was good for all samples analysed (croton oil-treated = 9-12, 6 h; 13-16, 24 h; untreated = 1-4, 6 h; 5-8, 24 h), as shown by the high RNA integrity number (RIN) and the presence of only two predominant bands corresponding to the ribosomal RNAs 28s and 18s. This translated into good quality data obtained by real-time PCR analysis; indeed, quantification of the transcripts of *Tbp* and *Gapdh* in ears revealed no difference between untreated (CTRL) and croton oil-treated groups either at 6 or 4 h (Figure 3.4 b and c).

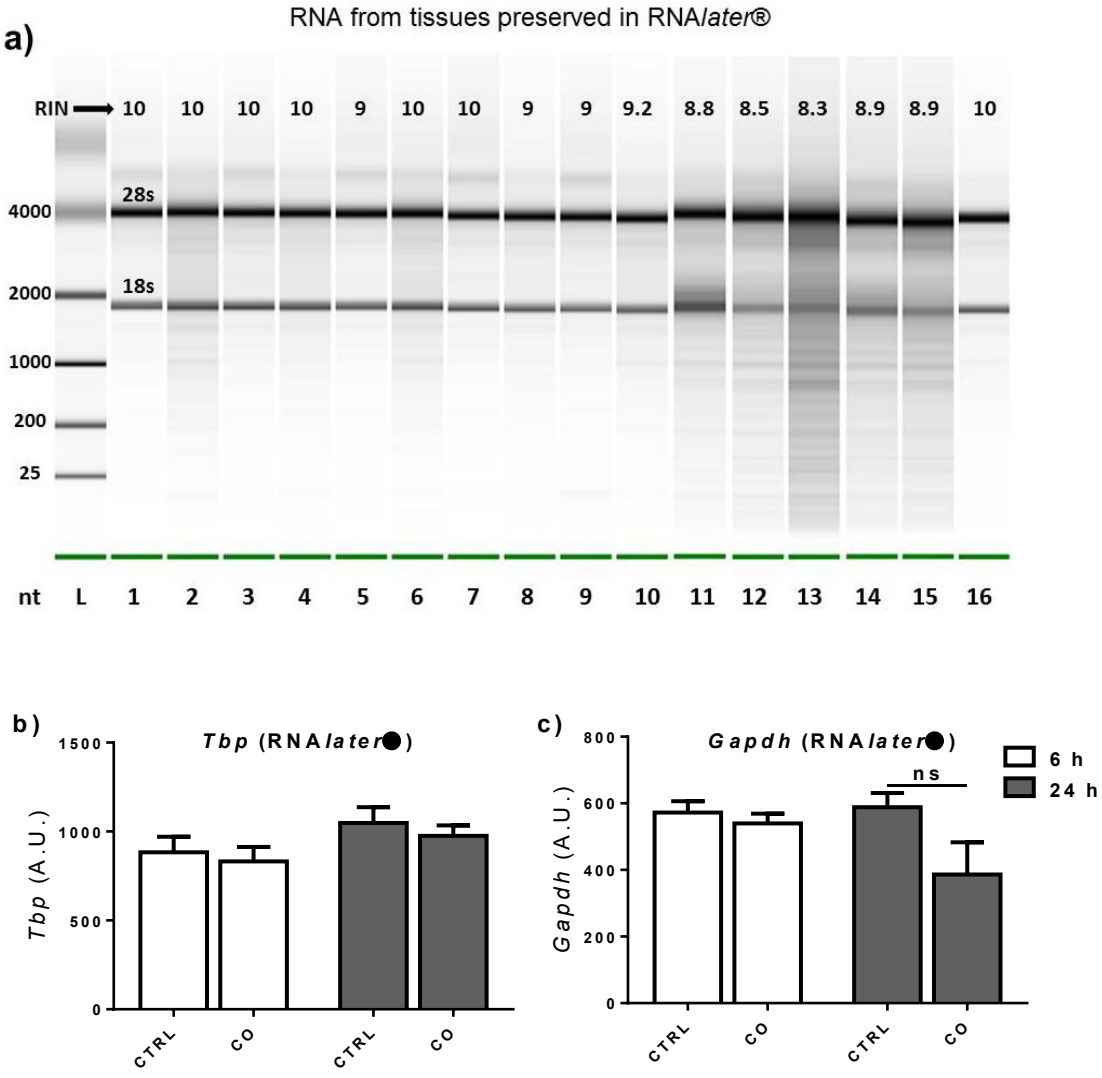

Figure 3.4 Collection of auricular tissues in RNA*later*® yielded good quality RNA. (a) Gel electrophoresis of either untreated (1-4, 6 hours (h); 5-8, 24 h) or croton-oil treated ears (9-12, 6 h; 13-16, 24 h) collected in RNA*later*® and stored at - 20°C; nt = number of nucleotides, L = RNA ladder, RIN = RNA integrity number, 28s (5070 nt) and 18s (1869 nt) = sizes of the correspondent ribosomal RNAs in Svedberg units. (b) and (c) Abundance of transcripts of TATA-binding protein (*Tbp*) and glyceraldehyde 3- phosphate dehydrogenase (*Gapdh*) respectively in control (CTRL) and croton (CO)-treated ears quantified through real-time PCR at 6 and 24 hours (h). Data (mean ± SEM) were analysed at each time point by unpaired Student's t-test; ns = not significant; n = 4/group. A.U. = Arbitrary Unit.

3.3.1.4 Abundance of transcripts of pro-inflammatory genes in inflamed ears

The abundance of transcripts of *Il6*, *Il1β* and *Tnfα* increased after treatment with croton oil for 6 and 24 h compared with untreated ears (Figure 3.5). This increase was more pronounced for *Il6* compared with *Il1β* and *Tnfα*, at both time points, and was higher at 6 compared with 24 h for all three genes.

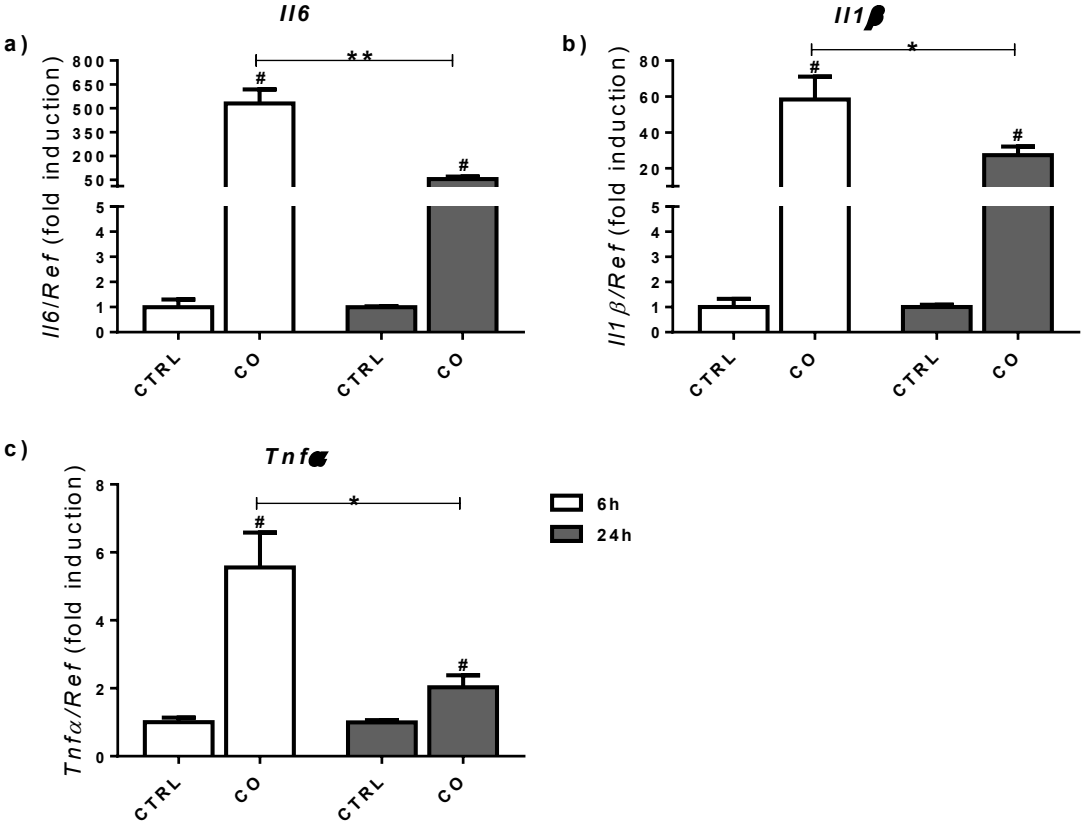

Figure 3.5 Treatment with croton oil (CO) for 6 and 24 hours (h) increased abundance of transcripts of pro-inflammatory genes. Abundance of transcripts of (a) *Il6*, (b) *Il1β* and (c) *Tnfα* represented as fold induction versus untreated ears. CTRL = control ears. Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed using unpaired Student's t-tests; # = $p < 0.05$ vs CTRL; ** = $p < 0.001$, * = $p < 0.05$; n=4-6/group.

3.3.2 Efficacy of B to suppress irritant dermatitis

The next step was to test how the model would respond to applications of B. Increasing concentrations of B reduced the extent of swelling (Figure 3.6 a and b) in a dose-dependent manner after 6 and 24 h from induction. The EC_{50} for B at 6 h-post treatment was 10 μg, while at 24 h it was 5 μg.

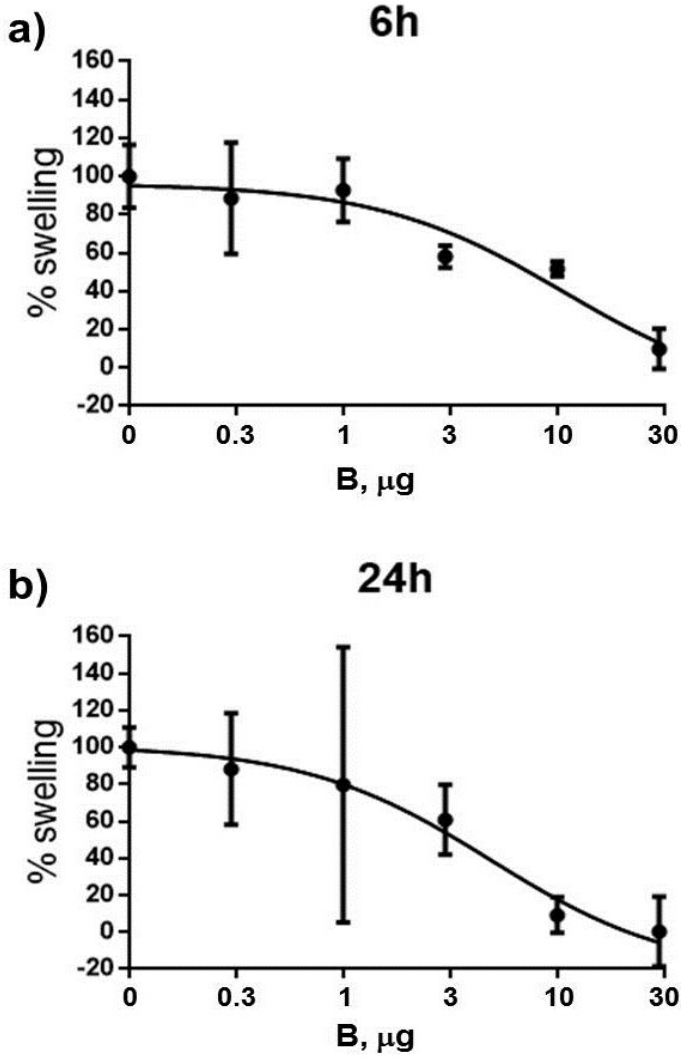

Figure 3.6 Corticosterone (B) reduced croton oil-induced inflammatory swelling in a dose-dependent manner. Reduction in inflammatory swelling by increasing concentration of B after (a) 6 and (b) 24 hours (h) of treatment with croton oil. 6 h, n=4/group; 24h, n=8-12/group. Data are mean ± SEM. The graph was drawn as a semi-log plot; the log values were substituted with the real concentrations of B to give a clearer representation.

3.3.3 Efficacy of 5α-THB to alleviate irritant dermatitis in mice

Having established a suitable protocol to demonstrate the anti-inflammatory properties of glucocorticoids to suppress ear inflammation, the model was then used to explore the actions of 5α-THB in comparison to B.

3.3.3.1 Effect of 5α-THB to alleviate inflammatory swelling

The efficacy of 5α-THB to reduce inflammatory swelling of the ear in comparison with B at 6 h was studied by applying a dose of B corresponding to the EC_{50} (10 μg), whereas 5α-THB was applied at 10, 30 and 50 μg. In these conditions, B reduced inflammation to 35.5 ± 8.7% compared with the croton oil-treated group (set to 100%), while an effect was not observed with 5α-THB at any concentration tested (Figure 3.7 a).

The efficacy of 5α-THB to reduce swelling induced by treatment with croton oil lasting 24 h was studied by applying B at its EC_{50} (5 μg), while 5α-THB was applied at 5, 15 and 25 μg (EC_{50} from previous studies (Livingstone, Sykes et al. 2014)). B reduced swelling to 57.0 ± 4.5%; 5α-THB decreased it to 85.3 ± 11.3% at 5μg, to 64.3 ± 15.7% at 15 μg and to 64.9 ± 7.9% at 25 μg (3.7 b) compared with the croton oil-treated group (set to 100%). Only the effect induced by 25 μg was statistically significant compared with the group treated with croton oil alone. At 15 μg a trend toward significance was found.

Subsequent analysis of samples treated with 5α-THB was restricted to tissues harvested following treatment with the highest concentrations used (50 μg at 6 h and 25 μg at 24 h).

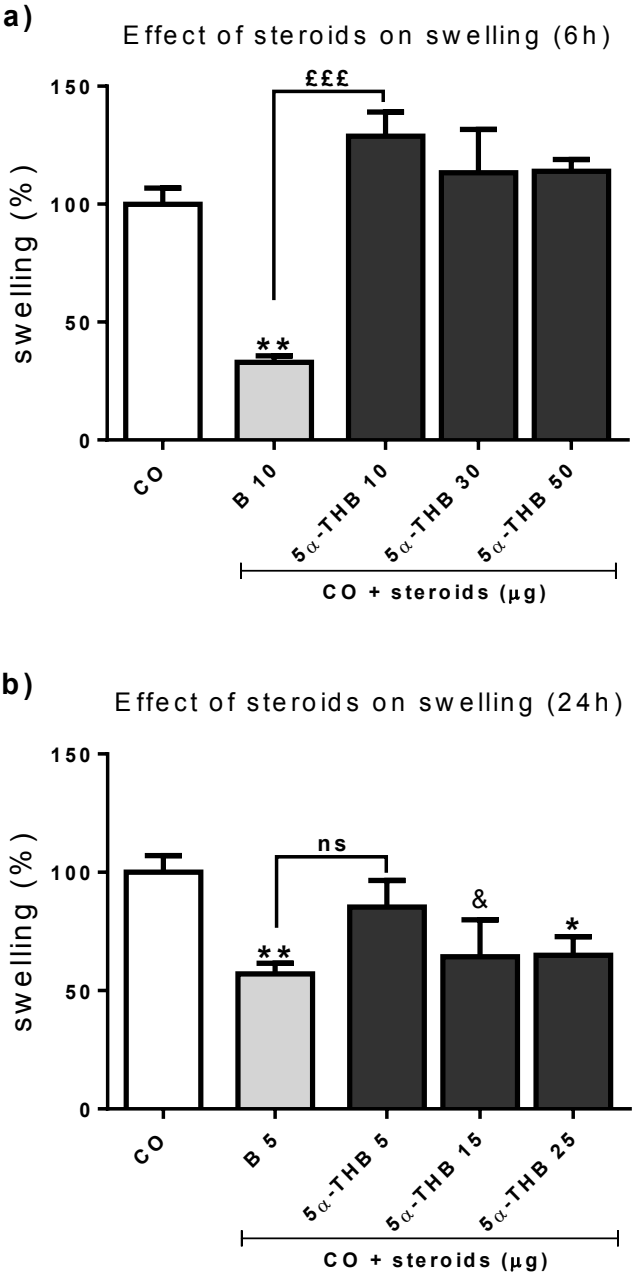

Figure 3.7 5α-Tetrahydrocorticosterone (5α-THB) suppressed swelling induced by treatment with croton oil (CO) lasting 24 but not 6 hours (h). Efficacy of 5α-THB and corticosterone (B) to suppress swelling induced by treatment with CO for (a) 6 and (b) 24 h. Data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; ** = $p < 0.001$, * = $p < 0.05$, & = $0.05 < p < 0.1$ vs CO; £££ = $p < 0.0001$; ns = not significant. 6h, n=4-8/group; 24h, n = 5-19/group.

3.3.3.2 Histological analysis of the effect of 5α-THB to suppress swelling and cell infiltration

Ear samples were analysed microscopically in order to compare the effect of co-application of croton oil and steroids for 6 or 24 h on swelling and cell infiltration.

3.3.3.2.1 Analysis of ears treated for 6 h

Treatment with croton oil for 6 h caused swelling of the tissue as clearly seen by the increase in the width of the dermal layer (Figure 3.8 A, B). B (10 μg), but not 5α-THB (50 μg), reduced swelling as seen from the reduction of width of the dermal layer (Figure 3.8 C, D) and confirmed by the quantification of the dermal thickness (Figure 3.9 a).

Counts of cell nuclei in the tissue (black arrows in Figure 3.8 A-D) revealed an increase in the number of cells infiltrating the ear after application of croton oil (Figure 3.9 b). B, but not 5α-THB, decreased the number (Figure 3.9 b).

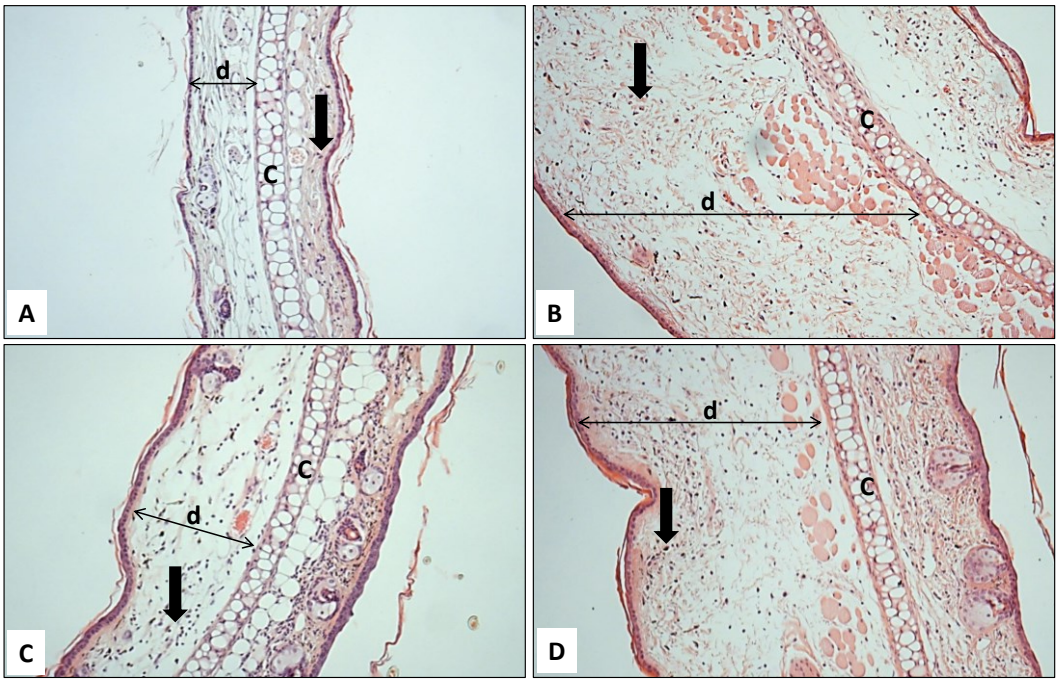

Figure 3.8 5α-Tetrahydrocorticosterone (5α-THB) did not reduce swelling induced by croton oil when applied for 6 hours (h). Representative images of ear tissue stained with haematoxylin and eosin showing changes in the width of dermal layer after treatment with croton oil alone or together with steroids for 6 h. (A) untreated ear, (B) ear treated with croton oil only, (C) ear co-treated with croton oil and B (10 μg), (D) ear co-treated with croton oil and 5α-THB (50 μg). Arrows point to cell nuclei of infiltrating cells; c=cartilage; d = dermis, which can be found at both sides of the ear cartilage; only one dermal layer was highlighted for clarity. Magnification = 100x.

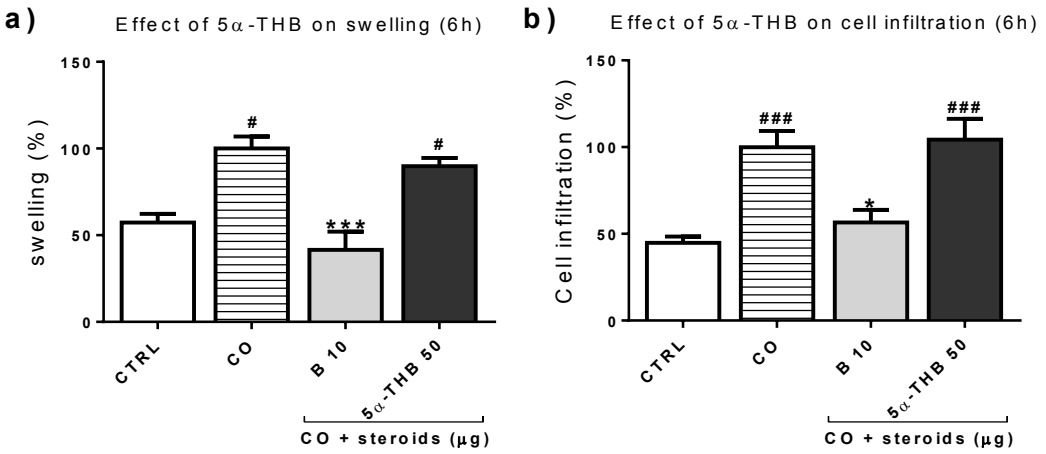

Figure 3.9 5α-Tetrahydrocorticosterone (5α-THB) did not reduce swelling and cell infiltration induced by application of croton oil (CO) for 6 hours (h). Quantitative measurement of (a) dermal thickness and (b) cell infiltration after treatment with CO alone or together with steroids for 6 h. CTRL = control ears; ### = $p < 0.0001$, # = $p < 0.05$ vs CTRL;*** = $p < 0.0001$, * = $p < 0.05$ vs CO; data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; n=6-14 tissue sections/group.

3.3.3.2.2 Analysis of ears treated for 24 h

Treatment with croton oil for 24 h caused swelling of the tissue as clearly seen by the increase in the width of the dermal layer (Figure 3.10 A, B). Both B (5 µg) and 5α-THB (25 µg) reduced swelling as seen from the reduction of width of the dermal layer (Figure 3.10 C, D) and confirmed by the quantification of the dermal thickness (Figure 3.11 a).

Counts of cell nuclei in the tissue (black arrows in Figure 3.10 A-D) revealed an increase in the number of cell infiltrating the ear after application of croton oil (Figure 3.11 b). B and 5α-THB decreased this number (Figure 3.11 b).

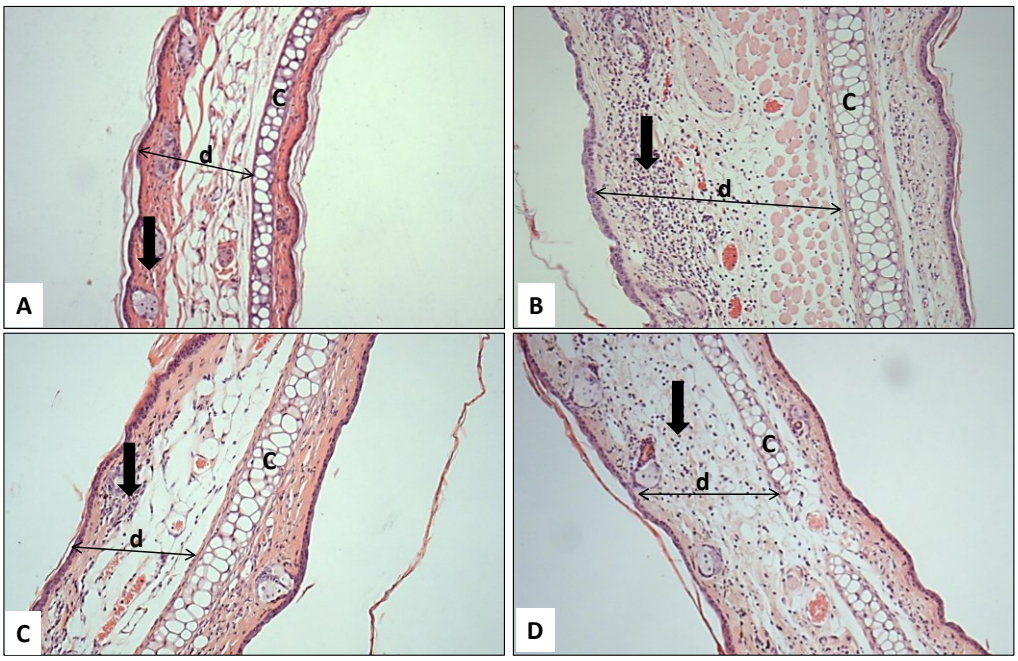

Figure 3.10 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) reduced swelling induced by croton oil when applied for 24 hours (h). Representative images of ear tissue stained with haematoxylin and eosin showing changes in the width of dermal layer after treatment with croton oil alone or together with steroids for 24 h. (A) untreated ear, (B) ear treated with croton oil only, (C) ear co-treated with croton oil and B (5 µg), (D) ear co-treated with croton oil and 5α-THB (25 µg). Arrows point to cell nuclei of infiltrating cells; c=cartilage; d = dermis, which can be found at both sides of the ear cartilage; only one dermal layer was highlighted for clarity. Magnification = 100x.

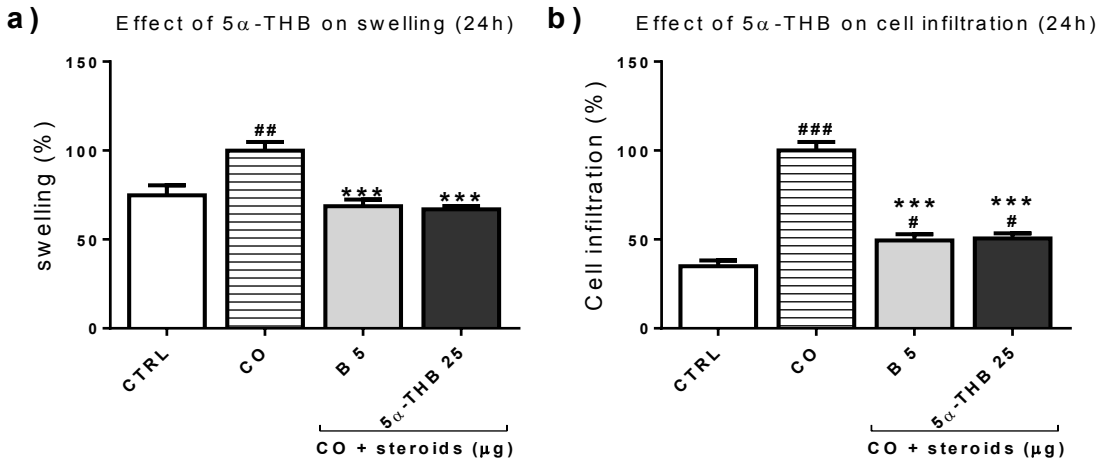

Figure 3.11 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) reduced swelling and cell infiltration induced by application of croton oil (CO) for 24 hours (h). Quantitative measurement of (a) dermal thickness and (b) cell infiltration after treatment with CO alone or together with steroids for 24 h. CTRL = control ears; ### = $p < 0.0001$, # = $p < 0.05$ vs CTRL; *** = $p < 0.0001$ vs CO; data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; n=4-10 tissue sections/group.

3.3.3.3 Effect of 5α-THB on infiltration of polymorphonuclear (PMN) cells

Qualitative analysis of samples collected after 6 and 24 h of treatment with croton oil and stained with H&E showed a pool of inflammatory cells in the tissue and blood vessels. The majority of these cells displayed a nucleus with a multi-lobulated shape (Figure 3.12 A and B, black arrows), a presentation which is typical of PMN cells of which neutrophils represent the highest percentage. In order to quantify this pool and to investigate the effect of co-application of steroids, the activity of the enzyme myeloperoxidase (MPO), which is most abundantly expressed in neutrophils, was assessed.

Application of croton oil for 6 and 24 h increased MPO activity compared with untreated ears; this increase was bigger at 6 h compared with 24 h. Co-application of B (10 and 5 μg) reduced this increase at both time points while 5α-THB (25 μg) did so only when co-applied for 24 h (Figure 3.13 a-b). At this time point the effect of 5α-THB was stronger than that of B (Figure 3.13 b).

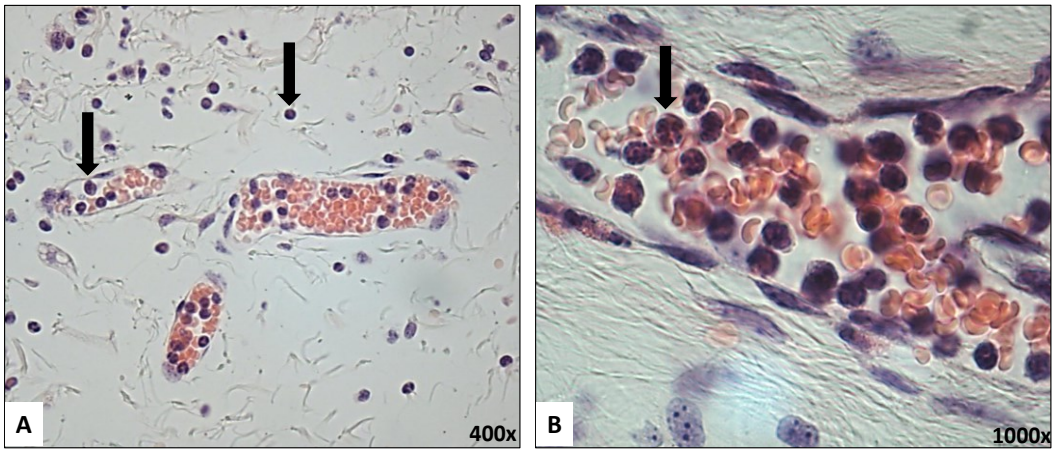

Figure 3.12 Croton oil application stimulated infiltration of polymorphonuclear (PMN) cells in ears. Representative images of dermal tissue and vessels of ears treated with croton oil and stained with haematoxylin and eosin showing PMN cell infiltration (black arrow). In this particular example the treatment lasted for 6 h. Magnification: A = 400X; B = 1000X.

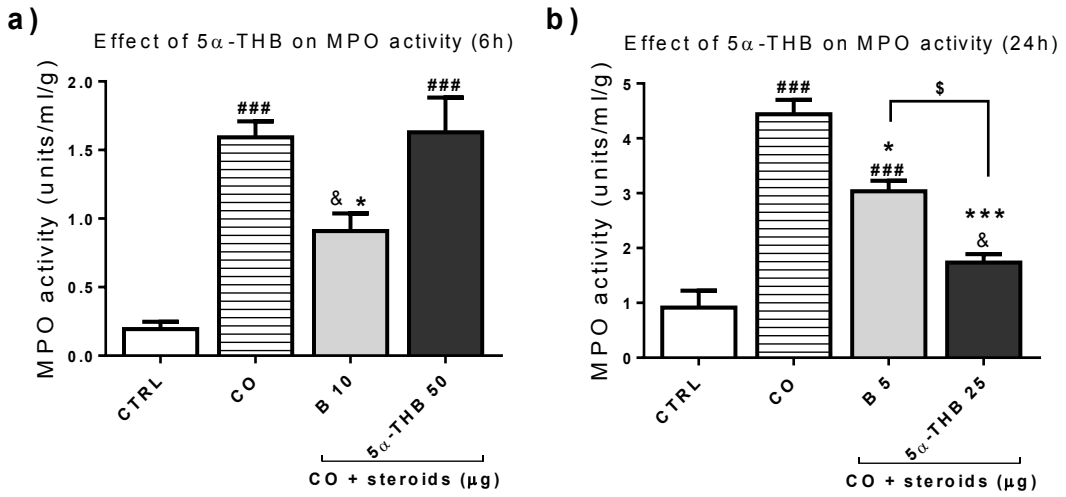

Figure 3.13 Croton oil (CO)-induced increase of myeloperoxidase (MPO) activity in ears was decreased by application of 5α-tetrahydrocorticosterone (5α-THB) for 24 but not 6 hours (h). Quantification of MPO activity in auricular tissues treated either with CO alone or in combination with corticosterone (B) or 5α-THB at the concentration indicated; CTRL = control ears. ### = $p < 0.0001$, & = $0.05 < p < 0.1$ vs CTRL; *** = $p < 0.0001$, * = $p < 0.05$ vs CO; $ = $p < 0.05$. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; n=8-10/group.

3.3.3.4 Effect of 5α-THB on abundance of transcripts of genes involved in the local inflammatory response

The effect of 5α-THB on abundance of transcripts for pertinent genes was investigated by real-time PCR only using ears from mice treated for 24 h, as this was the time point at which the compound showed anti-inflammatory properties.

Treatment with croton oil alone increased the abundance of transcripts of the pro-inflammatory genes *Il6*, *Tnfα*, *Il1β* and *Infγ* compared with untreated ears (Figure 3.14 a-d). Of these, only the increase in *Infγ* transcripts was reduced by co-treatment with B or 5α-THB (Figure 3.14 d). Application of croton oil alone increased also the transcripts for the anti-inflammatory gene *Dusp1* compared with untreated tissues (Figure 3.14 e). Interestingly, 5α-THB, but not B, increased further the abundance of *Dusp1* transcripts compared with tissue treated only with croton oil (Figure 3.14 e). Treatment with croton oil did not affect the abundance of transcripts of the anti-inflammatory gene Annexin A1; B and 5α-THB increased the abundance compared with tissues treated only with croton oil (Figure 3.14 f).

The abundance of transcripts of genes controlling the permeability of the vasculature and infiltration of inflammatory cells was also studied. Treatment with croton oil alone increased the abundance of transcripts of all genes compared with untreated ears (Figure 3.15 a-f). Both B and 5α-THB decreased, to a similar extent, the amount of mRNAs of *Vegfα*, *Pecam1*, *Icam1* and *VE-cadherin* (Figure 3.15 a, c-e) compared with the croton oil-treated group, but did not affect *E-selectin* or *Mcp1* (Figure 3.15 b and f).

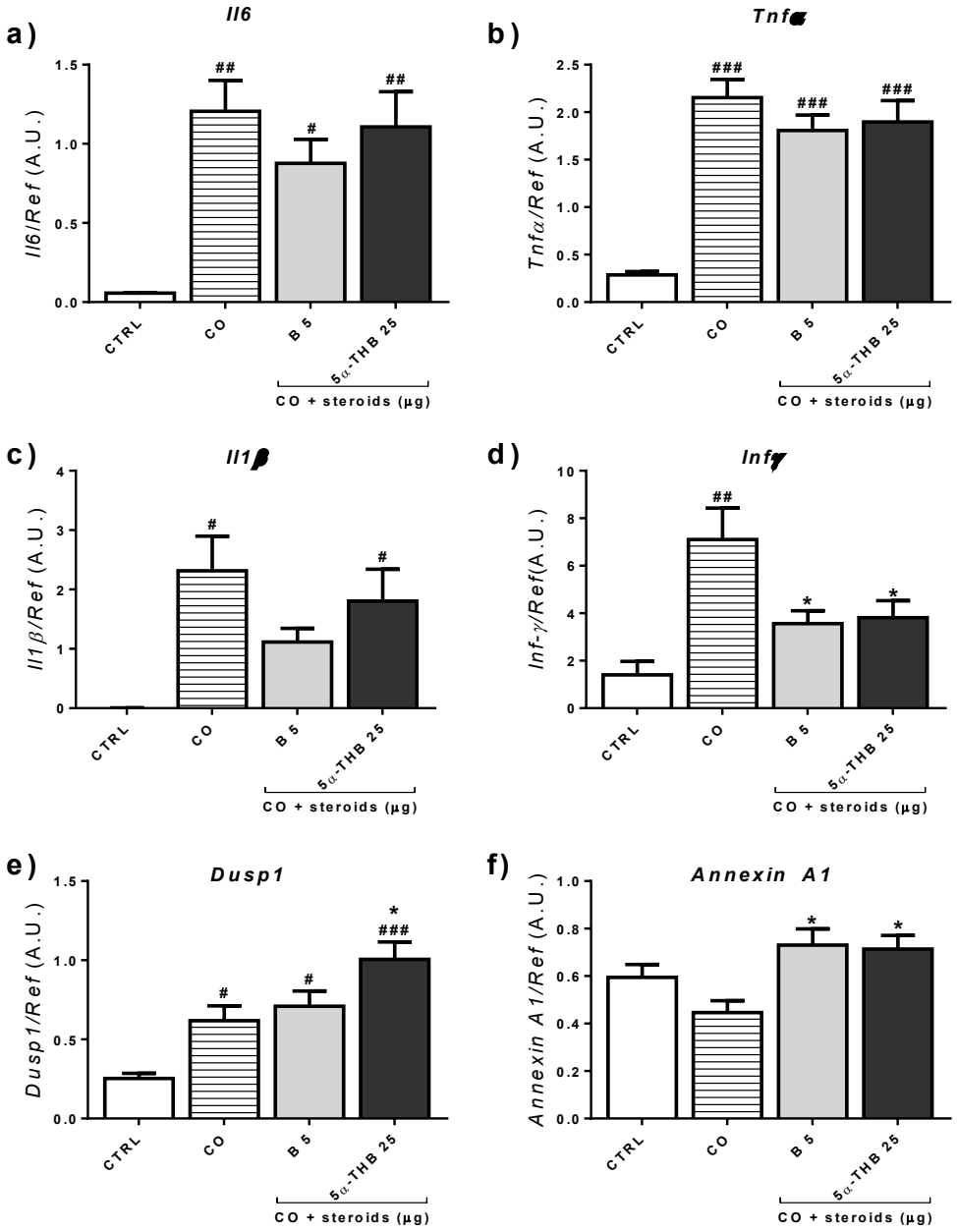

Figure 3.14 5α-Tetrahydrocorticosterone (5α-THB) increased the abundance of transcripts of the anti-inflammatory gene *Dusp1* in inflamed ears. Real-time analysis of the abundance of transcripts of the pro-inflammatory genes (a) *Il6*, (b) *Tnfα*, (c) *Il1β* and (d) *Infγ*, and the anti-inflammatory genes (e) *Dusp1* and (f) *Annexin A1*. CTRL = control ears; CO = croton oil; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ### = $p < 0.0001$, ## = $p < 0.001$, # = $p < 0.05$ vs CTRL; * = $p < 0.05$ vs CO; n=10/group.

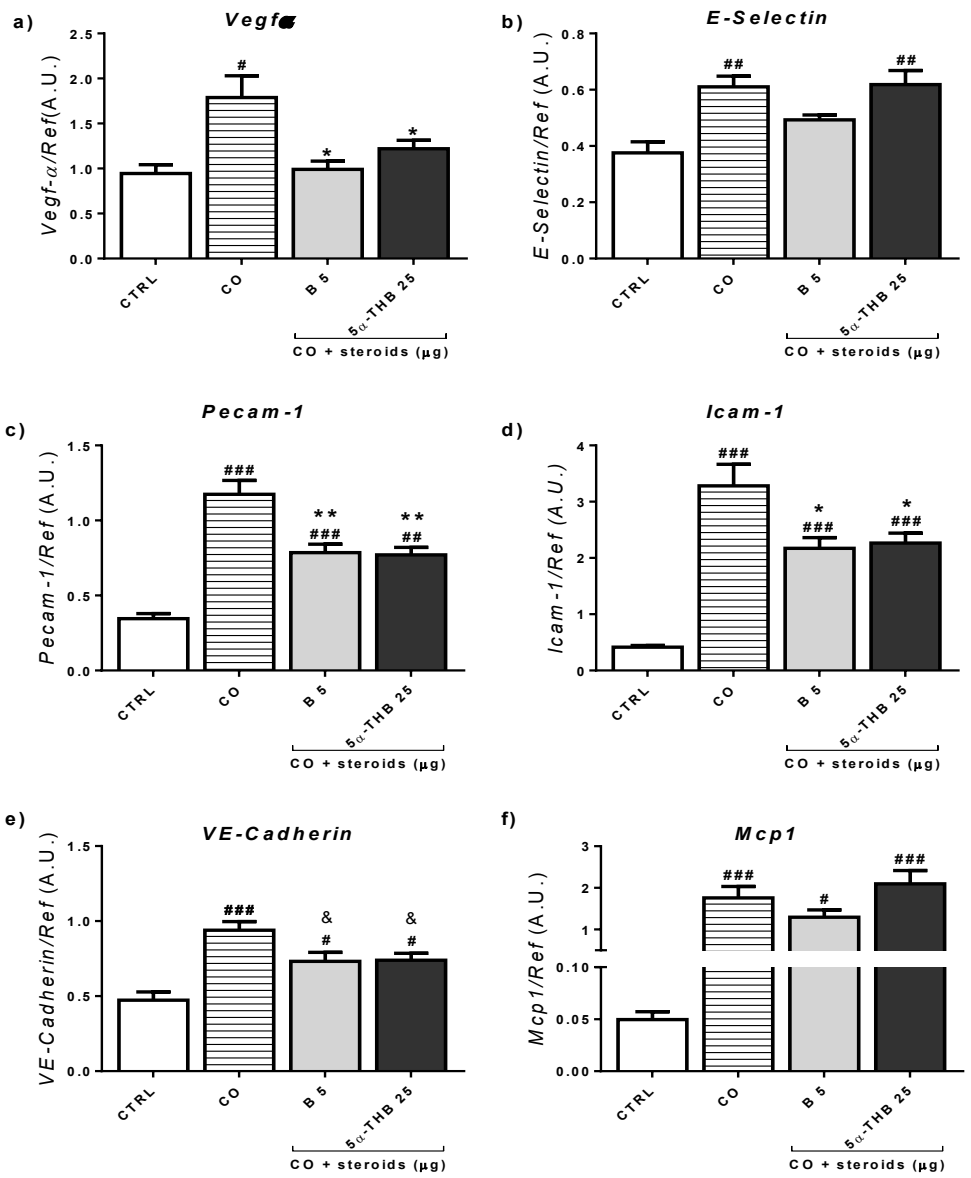

Figure 3.15 In inflamed ears 5α-tetrahydrocorticosterone (5α-THB) and corticosterone (B) similarly decreased the abundance of transcripts of genes involved in the control of vascular permeability. Real-time analysis of the abundance of transcripts of the vascular and inflammatory genes (a) *Vegfα*, (b) *E-selectin*, (c) *Pecam1*, (d) *Icam1*, (e) *Ve-cadherin* and (f) *Mcp1*. CTRL = control ears; CO = croton oil; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ### = $p < 0.0001$, ## = $p < 0.001$, # = $p < 0.05$ vs CTRL; ** = $p < 0.001$, * = $p < 0.05$ vs CO; & = $0.05 < p < 0.1$ vs CO; n=10/group.

During inflammation tissues go through a process of remodelling to allow the recovery of homeostasis and the healing process to take place. These processes are in part dependent upon enzymes such as metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Treatment with croton oil increased the abundance of transcripts of *Mmp2*, *Timp2*, *Mmp9* and *Mmp10* compared with untreated ears (Figure 3.16 a-d). 5α-THB did not affect the abundance of any of the transcripts, while B decreased the amount of mRNAs of *Mmp9* compared with the group treated only with croton oil (Figure 3.16 c).

In response to the action of MMPs and TIMPS, and to the inflammatory process itself, structural changes take place in the extracellular matrix to allow, for example, for the movement of inflammatory cells and for the recovery of the physiological architecture of the tissue after the damage. The abundance of transcripts of genes encoding structural proteins was, therefore, examined. Application of croton oil alone increased the abundance of transcripts for all genes investigated: keratin 6, actin and collagen 4a1 (Figure 3.17 a-c). B suppressed this increase for all of them (Figure 3.17 a-c), whereas 5α-THB did so only for actin and collagen 4a1 (Figure 3.17 b, c).

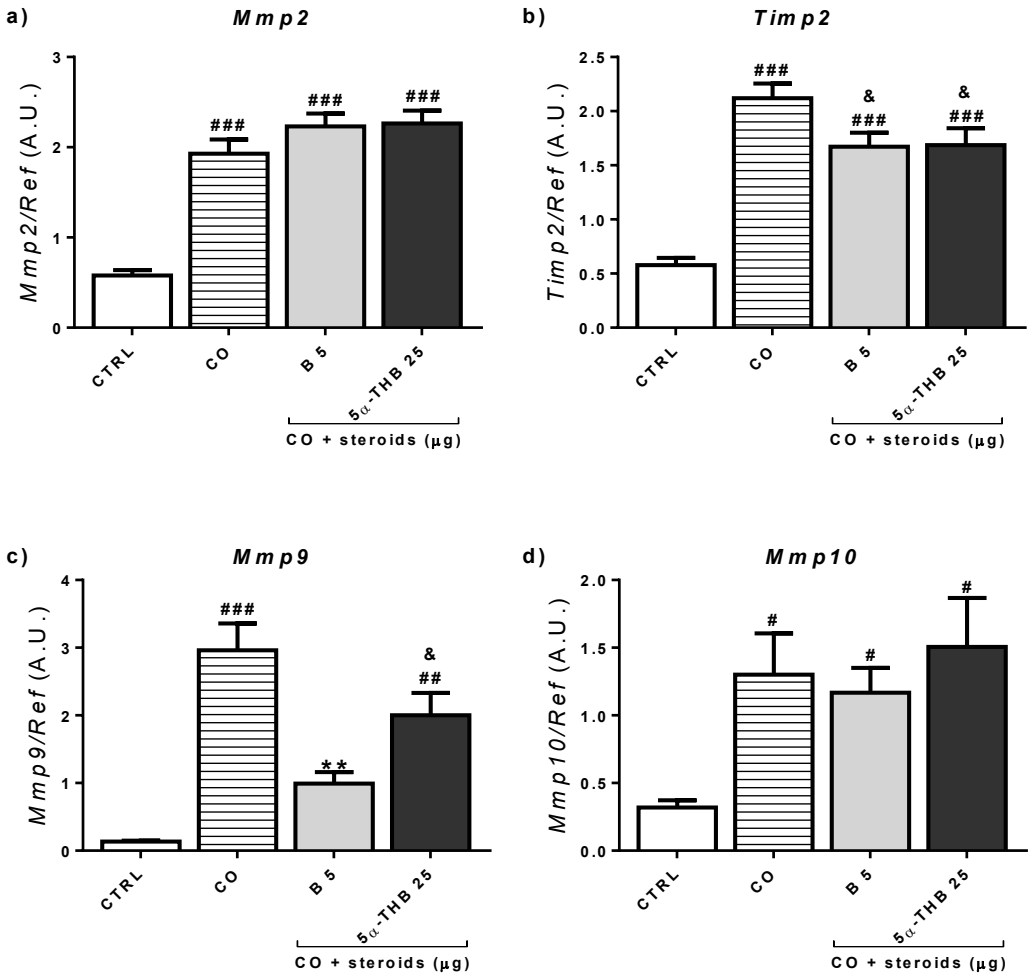

Figure 3.16 In inflamed ears 5α-tetrahydrocorticosterone (5α-THB) did not affect the abundance of transcripts of genes involved in remodelling of the extracellular matrix (ECM). Real-time analysis of the abundance of transcripts of (a) *Mmp2*, (b) *Timp2,* (c) *Mmp9* and (d) *Mmp10*. CTRL = control ears; CO = croton oil; Ref = average of two housekeeping genes, B = corticosterone. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ### = $p < 0.0001$, ## = $p < 0.001$, # = $p < 0.05$ vs CTRL; ** = $p < 0.001$, & = $0.05 < p < 0.1$ vs CO; n=10/group.

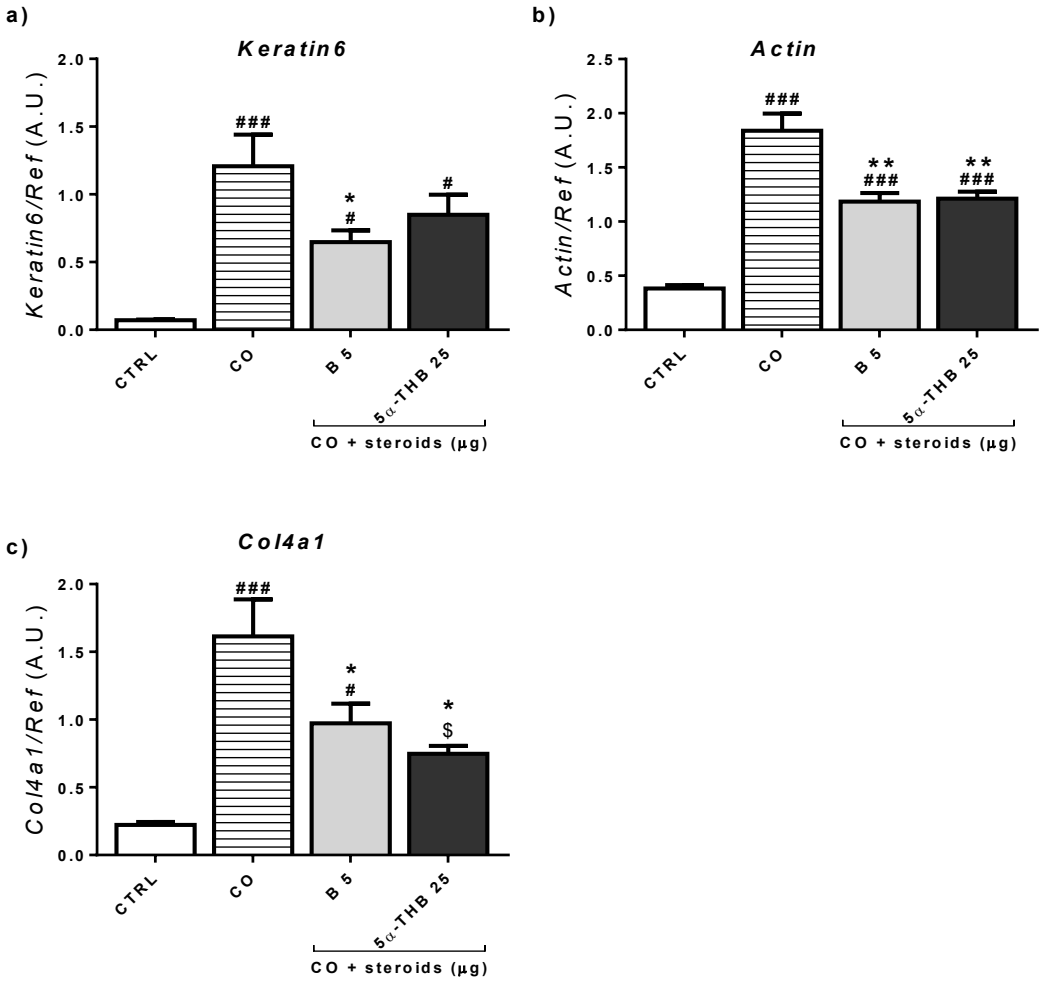

Figure 3.17 In inflamed ears 5α-tetrahydrocorticosterone (5α-THB) decreased the abundance of transcripts of *Col4a1* and *Actin*. Real-time analysis of the abundance of transcripts of (a) keratin 6, (b) actin and (c) *Col4a1*. CTRL = control ears; CO = croton oil; Ref = average of two housekeeping genes, B = corticosterone. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ### = $p < 0.0001$, # = $p < 0.05$ vs CTRL; ** = $p < 0.001$, * = $p < 0.05$ vs CO; $ = $0.05 < p < 0.1$ vs CTRL; n=10/group.

3.3.4 Are the anti-inflammatory effects of 5α-THB dependent on the glucocorticoid receptor (GR)?

3.3.4.1 Development of a model

3.3.4.1.1 Topical application of RU486

In order to investigate whether the anti-inflammatory effect of 5α-THB on the croton oil model of irritant dermatitis is mediated by GR, the antagonist RU486 was used. Firstly, a working method to deliver the compound needed to be developed. To begin with, RU486 was applied topically on the ears of mice in conjunction with croton oil and B for 24 h. Attenuation of the effects of B was considered a positive control.

As seen in Figure 3.18, croton oil application caused swelling of the ear, while B decreased it. Topical application of RU486 did not diminish the anti-inflammatory effect of B.

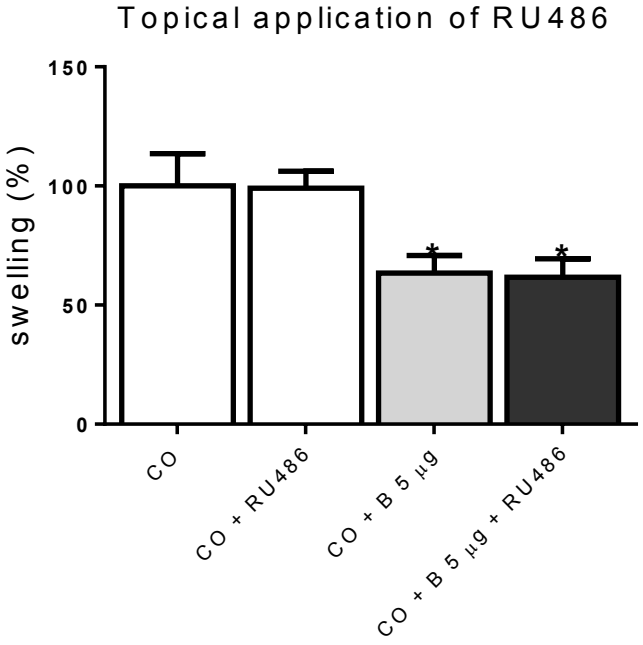

Figure 3.18 Topical application of RU486 did not prevent the anti-inflammatory effect of corticosterone (B) on swelling induced by croton oil (CO). Quantification of the effect of B alone or together with RU486 (6.2 µg) on the inflammatory swelling induced by the application of CO for 24 hours. Data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; * = $p < 0.05$ vs CO; n=6-10/group.

3.3.4.1.2 Subcutaneous injection of RU486

Subcutaneous injection of a solution of RU486 in DMSO was tested as an alternative method to deliver the drug. As seen in Figure 3.19, injection of the vehicle (DMSO) alone reduced the swelling induced by application of croton oil for 24 h, independently from co-treatment with B, and injection of RU486 alleviated this effect also independently from the presence of B.

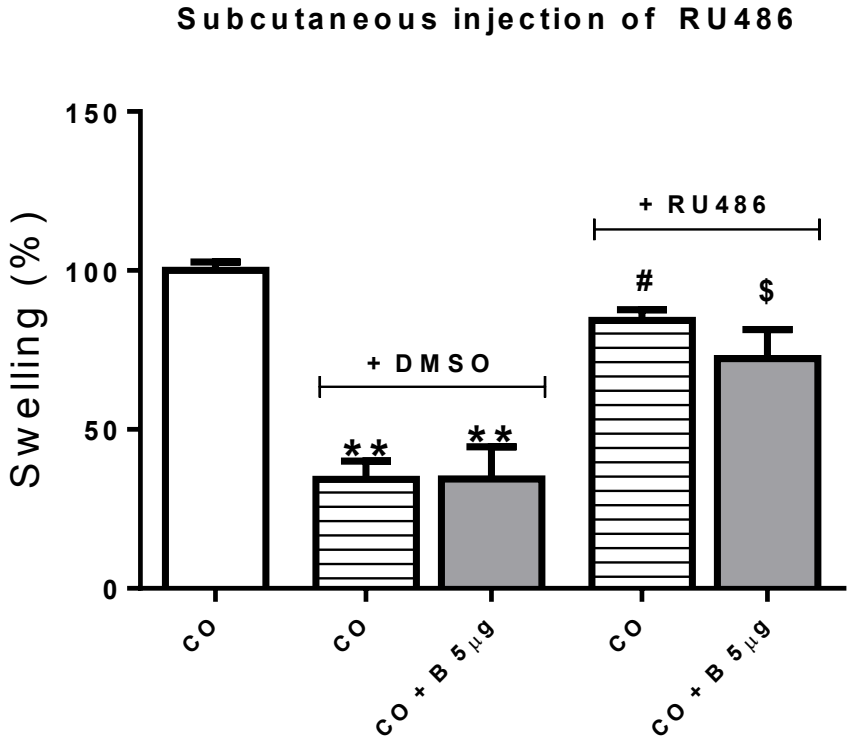

Figure 3.19 Subcutaneous injection of dimethyl sulfoxide (DMSO) reduced swelling in inflamed ears independently from treatment with corticosterone (B), and injection of RU486 alleviated this effect. Quantification of the inflammatory swelling in ears after either treatment with croton oil (CO) alone or injection of DMSO or RU486 prior to application of croton oil alone or together with B for 24 hours. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ** = $p < 0.001$ vs CO; # = $p < 0.05$ vs CO+DMSO; $ = $p < 0.05$ vs CO + B 5μg + DMSO; n=4/group.

3.3.4.1.3 Optimisation of the model

The results above indicated that the injection itself caused a reduction in the swelling induced by croton oil. This may have been an effect due either to the release of endogenous corticosterone in response to stress, or to the intrinsic anti-inflammatory properties of the vehicle DMSO, which have been documented before in this model (Coruzzi, Pozzoli et al. 2011) and others within the department (Dr. Mark Miller, personal communication), or to a combination of these two factors. As a consequence, two simultaneous measures were taken to address these concerns, by performing the experiment in adrenalectomised (ADX) mice and administering RU486 dissolved in ethanol.

Firstly, ADX mice were tested for their response to treatment with croton oil alone for 24 h in comparison with non-ADX mice. The extent of the inflammatory swelling between ADX and non-ADX mice was not different (Figure 3.20 a). When ADX mice were co-treated with croton oil and B, the steroid alleviated the swelling (Figure 3.20 b). These results showed that this was a suitable working model in which to test the effect of ethanolic RU486 on the anti-inflammatory properties of 5α-THB.

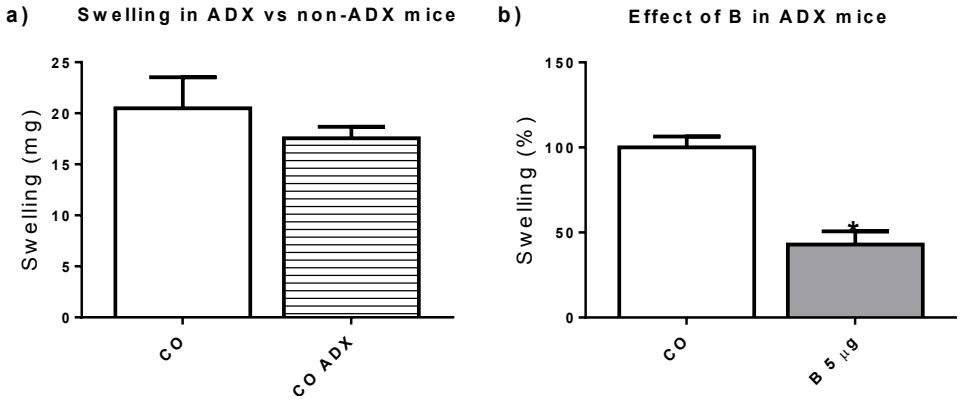

Figure 3.20 In adrenalectomised (ADX) mice croton oil (CO) caused ear swelling to a similar extent to non-ADX animals, and this inflammatory response was alleviated by corticosterone (B). (a) Quantification of inflammatory swelling (mg) after application of CO alone in ADX (CO ADX) and non-ADX (CO) mice. (b) Quantification of the anti-inflammatory effect of B in ADX mice treated with CO. Data (mean ± SEM) were analysed with unpaired Student's t-test; * = $p < 0.05$; n=4/group.

As shown in Figure 3.21, subcutaneous injection of ethanol alone or ethanolic RU486 did not have any effect on swelling induced by croton oil applied for 24 h, indicating that ethanol was a suitable solvent to use.

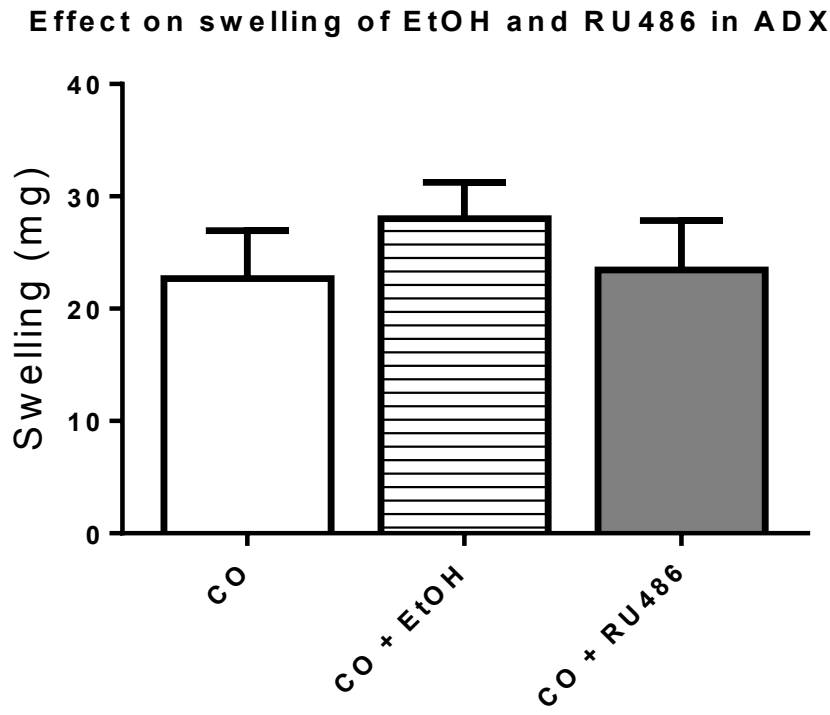

Figure 3.21 Injection of ethanol (EtOH) or ethanolic RU486 did not affect swelling induced by croton oil (CO) in adrenalectomised (ADX) mice. Quantification of ear swelling (in mg) after application of either CO alone or after injection of either ethanol or ethanolic RU486 (6.2 μg/mouse). Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; n=7/group.

3.3.4.2 Effect of RU486 on the anti-inflammatory properties of 5α-THB

The developed model was used to test the effect of injection of ethanolic RU486 on the reduction of swelling caused by 5α-THB after treatment for 24 h. As shown in Figure 3.22, B decreased croton oil-induced swelling, and the effect was attenuated by injection of RU486; 5α-THB lowered inflammatory swelling but in contrast to B, this effect was not affected by the administration of RU486.

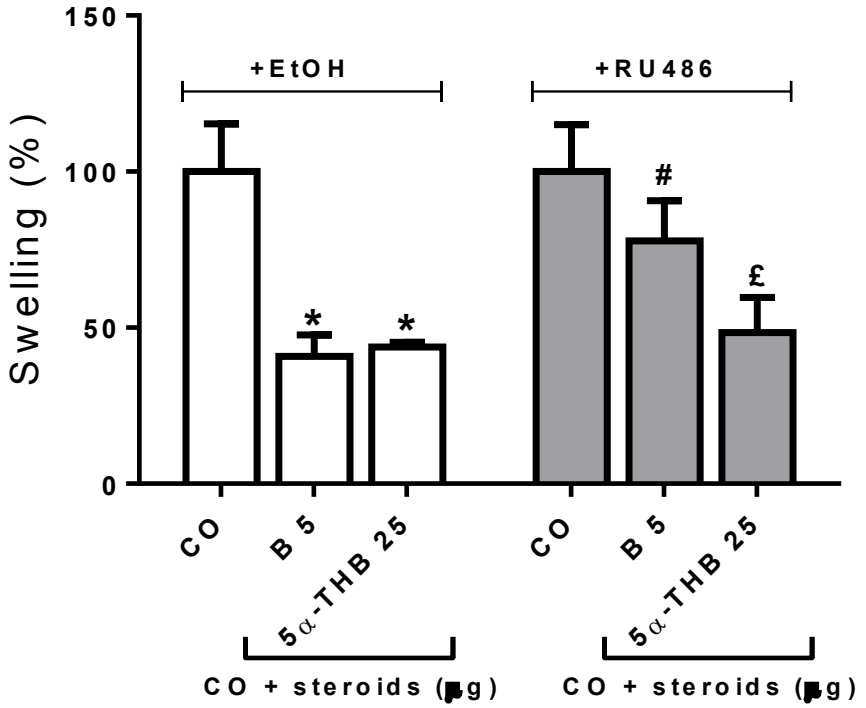

Figure 3.22 The anti-inflammatory effect of 5α-tetrahydrocorticosterone (5α-THB) on swelling was not attenuated by the subcutaneous injection of RU486. Quantification of ear swelling after injection of either ethanol (EtOH) or ethanolic RU486 prior to treatment with either croton oil (CO) alone or together with corticosterone (B) or 5α-THB for 24 hours. Data (mean ± SEM) were analysed using one-way ANOVA followed by Tukey's post-hoc test; * = $p < 0.05$ vs CO + EtOH; # = $p < 0.05$ vs CO + B5 + EtOH; £ = $p < 0.05$ vs CO + RU486; n=5-7/group.

3.3.5 Is the physiological production of 5α-THB important in the response to the treatment with croton oil and for the anti-inflammatory effects of steroidal substrates?

3.3.5.1 Analysis of the transcripts encoding 5α-reductase isozymes in ears

The presence of mRNAs encoding the two enzymes 5α-R1 and 5α-R2 in the ear was investigated first by PCR analysis. As shown in Figure 3.23 (a), a DNA band was present for both enzymes in the positive controls (liver for 5α-R1 and prostate for 5α-R2), while only 5α-R1 was detected in the ear sample. Analysis of the influence of the treatment with croton oil alone and in combination with steroids on the abundance of transcripts of *Srd5a1* (5α-R1) was also performed by real-time PCR analysis (Figure 3.23 b). Application of croton oil decreased the abundance of mRNAs of *Srd5a1*, whilst treatment with B or 5α-THB did not affect this change.

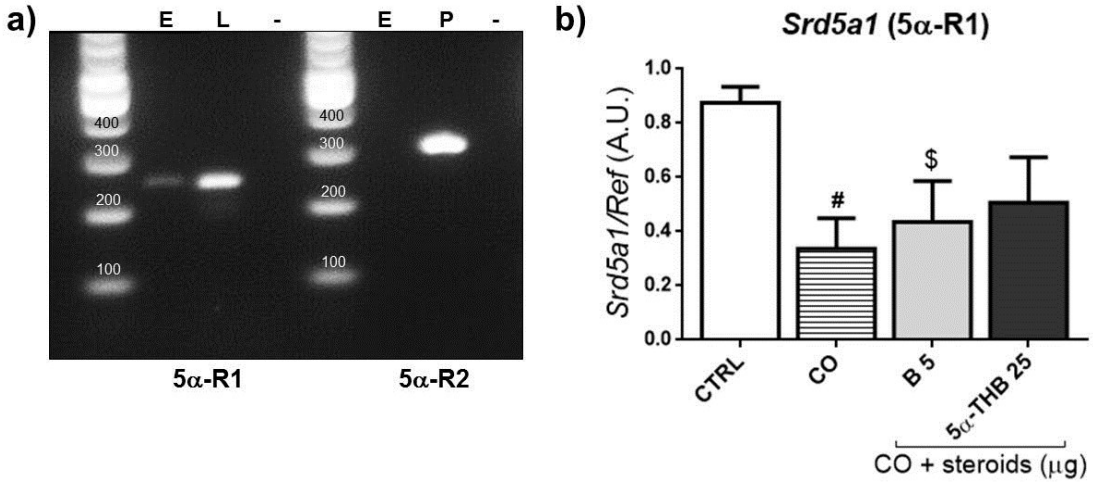

Figure 3.23 Transcripts for 5α-reductase type 1 (5α-R1), but not 5α-reductase type 2 (5α-R2), were present in ear tissue, and their abundance was decreased by the application of croton oil (CO) alone. (a) PCR analysis for 5α-R1 (product = 240 base pairs (bp)) and 5α-R2 (product = 299 bp) in ear (E), liver (L) and prostate (P) samples. Numbers represent the base pairs of the DNA ladder; the symbol (-) represents the negative controls. (b) Real-time analysis of transcripts of *Srd5a1*. CTRL = control ears; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; # = $p < 0.05$, $ = $0.05 < p < 0.1$ vs CTRL; n = 10/group.

Having verified the presence of transcripts for 5α-R1 in ear tissues, the inflammatory response induced by application of croton oil was then tested in mice with genetic disruption of the enzyme.

3.3.5.2 Effects of the genetic disruption of 5α-R1 on the concentration of B in plasma after treatment with croton oil

The concentration of B in plasma of mice with genetic disruption of 5α-R1 (5α-R1$^{-/-}$) and wild type littermates (WT), treated with croton oil for 6 or 24 h, was measured. No differences were found between the two groups (Figure 3.24).

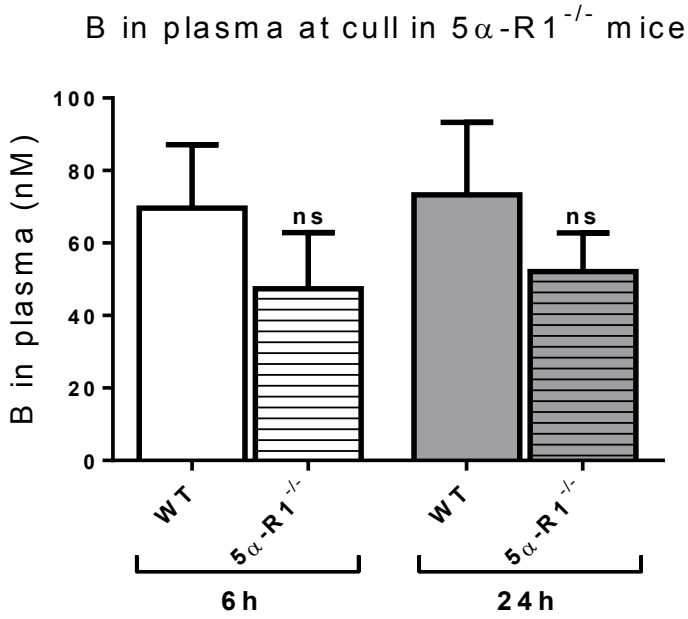

Figure 3.24 Plasma corticosterone (B) did not differ between mice with genetic disruption of 5α-reductase type 1 (5α-R1$^{-/-}$) and wild type littermates after treatment with croton oil. Quantification of the concentration of B in plasma (nM) by ELISA in mice treated with croton oil for 6 or 24 hours (h). WT = wild type littermates. Data (mean ± SEM) were analysed by unpaired Student's t-tests; ns = not significant; n = 6/group.

3.3.5.3 Analysis of the inflammatory response to application of croton oil in 5α-R1 $^{-/-}$ mice

The extent of the swelling was similar between 5α-R1$^{-/-}$ and WT mice, both after 6 and 24 h (Figure 3.25). The oedema was greater at 6 than at 24 h for both groups, reproducing what was shown previously in C57BL6 wild type mice (compare Figure 3.25 and Figure 3.1).

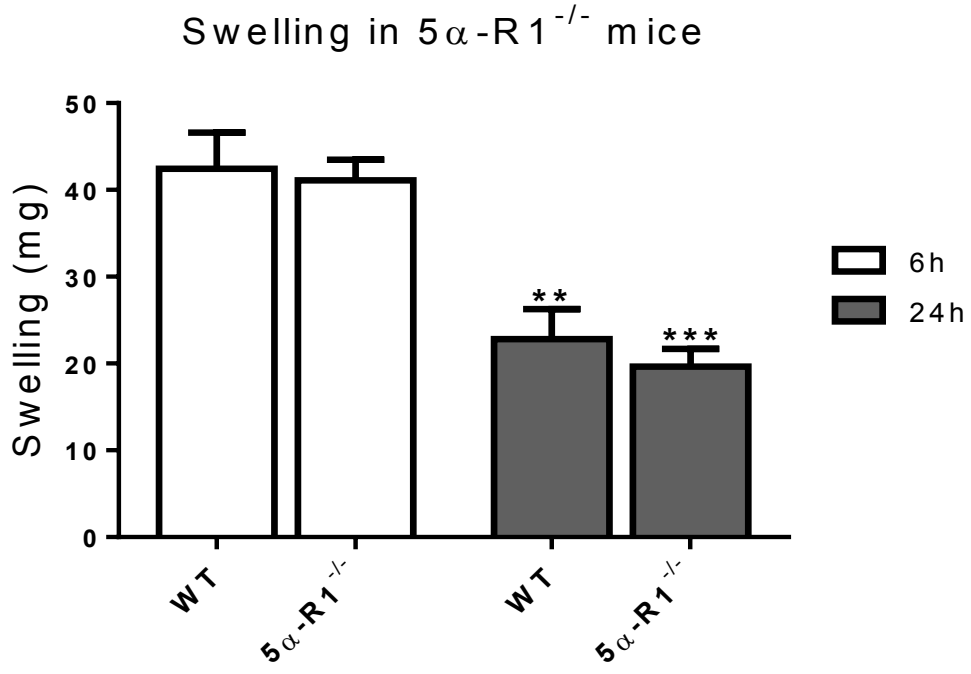

Figure 3.25 Swelling induced by treatment with croton oil (CO) for 6 and 24 hours (h) was not affected by the genetic disruption of 5α-reductase type 1 (5α-R1). Quantification of ear swelling (mg) induced by application of croton oil for either 6 or 24 h. WT = wild type littermates. Data (mean ± SEM) at each time point were analysed by unpaired Student's t-tests; *** = $p < 0.0001$ vs -/- 6h, ** = $p < 0.01$ vs WT 6h; n=6-8/group.

3.3.5.4 Efficacy of B and 5α-THB to suppress swelling in 5α-R1$^{-/-}$ mice

Both after 6 and 24 h of treatment, B (EC_{50}, 10 and 5 μg respectively) reduced the inflammatory swelling caused by croton oil application; the reduction was similar in the two genotypes (Figure 3.26 a, b).

As there is the possibility that the anti-inflammatory effects of 5α-THB could be due to its reversion back to B through the action of 5α-R1, its anti-inflammatory properties was tested at 24 h in 5α-R1$^{-/-}$ mice. As shown in Figure 3.26 b, 5α-THB decreased croton oil-induced swelling to the same extent in both genotypes.

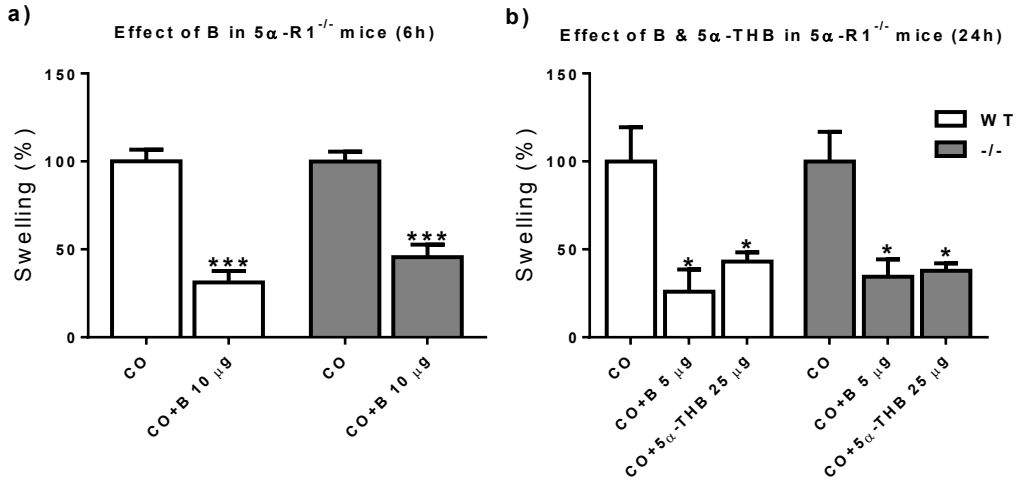

Figure 3.26 Genetic disruption of 5α-reductase type 1 (5α-R1) did not affect the ability of 5α-tetrahydrocorticosterone (5α-THB) and corticosterone (B) to reduce swelling induced by croton oil (CO). Quantification of swelling in 5α-R1$^{-/-}$ and wild type (WT) littermates mice after application of either CO alone or in combination with B or 5α-THB for (a) 6 or (b) 24 hours (h). Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc; *** = $p < 0.0001$, * = $p < 0.05$ vs CO; n=8-13/group.

3.4 Discussion

In this chapter the ability of 5α-THB to suppress inflammation was tested using the murine model of croton oil-induced irritant dermatitis through the analysis of a variety of molecular parameters. The main findings were that 5α-THB is anti-inflammatory, but compared with B is less potent and acts more slowly, being effective after 24 but not 6 h of treatment. At 24 h, 5α-THB reduced total cell infiltration in ear tissues and swelling to a similar extent to B. Interestingly, when the effect on infiltration of polymorphonuclear cells was studied, 5α-THB reduced the amount of this population more than B. At a molecular level, 5α-THB and B affected the same transcripts, mainly those encoding molecules involved in the control of vascular permeability; however, the RNA abundance of the anti-inflammatory gene *Dusp1* was induced significantly only by the 5α-reduced steroid. The most intriguing difference concerned the putative receptor targeted by the two steroids. While the anti-inflammatory action of B was prevented by the GR antagonist RU486, this did not happen for 5α-THB, suggesting that one the reasons for the selectivity of 5α-THB may be represented by the way it binds to GR, or by binding to a different receptor.

The application of croton oil to induce contact irritant dermatitis represents a research model that has been widely used in the literature (Gahring, Osborne et al. 2010), and it is a well-characterised steroid-responsive system. Croton oil is derived from the seeds of *Croton tiglium*, a tree originating from Asia. It is a mixture of lipids and contains irritant diester compounds that are derivatives of the diterpene phorbol, and that have both inflammatory and tumour-promoting activities (Hecker 1967; Tragni, Tubaro et al. 1985). Concerning the inflammatory properties, the damage caused by the mixture of chemicals applied to the skin promotes the release of cytokines and other molecules (i.e. IL1α and ATP) from keratinocytes and other local cells, which in turn stimulates the release of pro-inflammatory cytokines such as IL1β and IL6 from epidermal and dermal cells (McKenzie and Sauder 1990; Barker, Mitra et al. 1991; Effendy, Loffler et al. 2000; Lee, Stieger et al. 2013) causing swelling and infiltration of leukocytes. Research investigating the kinetics of

this inflammatory response (Tonelli, Thibault et al. 1965; Tubaro, Dri et al. 1986; Towbin, Pignat et al. 1995; Baumgartner, Sosa et al. 2011) showed that the predominant effect at the early stages of the process is tissue oedema which is at its peak at 6 h-post treatment; this phase is followed by one characterised by cell infiltration (mainly neutrophils) which is at its highest between 12 and 24 h, depending on the study. Most studies though have evaluated the inflammatory responses after treatment for 6 or 24 h, and therefore it was decided to test the effects of the interventions at these two time points. The data presented in this chapter are in accordance with the literature, therefore the choice of 6 and 24 h was deemed a good strategy in order to test how 5α-THB and B affected two different aspects of the inflammatory process. Furthermore, quantification of transcripts of pro-inflammatory genes showed a significant increase in groups treated with croton oil compared with untreated ears. The increase was greater at 6 compared with 24 h. In a preceding study (Towbin, Pignat et al. 1995), it was found that the abundance of IL1β protein was higher at 24 than 6 h, suggesting, as expected, that the kinetics of the production of the cytokine is slower than that of the transcripts.

Studies investigating the anti-inflammatory effect of GCs in the croton oil model of dermatitis have mostly used dexamethasone and other steroidal pharmaceutical formulations (i.e. HC) as positive controls, showing their potent inhibitory effects both on swelling and cell infiltration (Muramoto, Goto et al. 2010; Baumgartner, Sosa et al. 2011; da Silva, Sperotto et al. 2013; Vassallo, De Tommasi et al. 2013; Pacheco, Pinto Nde et al. 2014). To our knowledge, B has never been tested in this model; as a consequence, its potency and efficacy in reducing swelling was investigated before comparison with 5α-THB. Treatment with B induced a dose-dependent reduction of ear swelling, confirming the powerful anti-inflammatory properties of this endogenous glucocorticoid. The half maximal effective concentration of B, or EC_{50}, was calculated at 6 and 24 h and used in subsequent experiments. Using the EC_{50} allowed a more straightforward comparison between the two steroids as it is a measure of the potency of a drug. It was found that the EC_{50} at 6 h was double the EC_{50} at 24 (10 μg versus 5 μg). As already mentioned, the acute inflammation at the early stage (6 h) is mainly due to oedema of the tissue, while at

the later stage (24 h) it is predominantly the result of cell infiltration. The fact that B is efficacious at both time points indicates that both stages are affected by its action; however the fact that it is less potent at the early stage would suggest a stronger effect on cell infiltration. In previous unpublished studies from my supervisors' group, HC was used in the same model and the EC_{50} was found to be equivalent to approximately 13 μg at 24 h. The data presented here indicate that B is 2.6 times more potent than HC in this model.

Interestingly, 5α-THB significantly reduced ear swelling at 24 but not 6 h. Although the use of both 15 and 25 μg produced similar effects on oedema, only the higher concentration was statistically effective. The post-hoc comparison between the effect of the steroid at 15 μg and the croton oil group returned a p value of 0.07. This may be due to the higher variability of the data at this concentration, as suggested by the bigger standard error associated to the mean at 15 compared with 25 μg. This also indicates that because of this variability, the number of animals investigated should be increased in order to boost the statistical power of the analysis. Importantly though, at both concentrations, 5α-THB was as efficacious as B in reducing ear swelling making it three to five times less potent than B in this model, and therefore comparable to HC. In previous abovementioned studies the EC_{50} of 5α-THB was found to be 25 μg, at 24h. In this chapter the reduction in swelling caused by 5α-THB applied at its EC_{50} was not 50% but approximately 35%. Observations made during the execution of the experiments suggested that this mouse model is very susceptible to external changes, and the inflammatory response could be alleviated by any stressor. It is therefore possible that the change in the operator may have played a role in the response to the treatments. Some variability was seen also between experiments carried out by the same researcher. Nonetheless, 5α-THB was shown to be an effective anti-inflammatory agent.

Histological analysis showed that B was effective in reducing both swelling and cell infiltration at both time points, while 5α-THB reduced both parameters only at 24 h. The infiltration of polymorphonuclear (PMN) cells was analysed by performing the MPO activity assay. In the literature this measurement is taken to be representative

of the population of neutrophils infiltrating the tissue since they are the main cell type recruited to the site of injury in acute inflammation, and they represent the most abundant pool of cells among PMNs. More importantly, neutrophils have been shown to be the predominant cell type reaching the site of inflammation in this model (Gahring, Osborne et al. 2010). The results shown in this chapter confirmed what was seen microscopically and reported in the literature (Tubaro, Dri et al. 1986; Baumgartner, Sosa et al. 2011) in that these cells were barely detectable in control ears and increased in number following croton oil treatment, being more abundant at 24 than at 6 h. Regarding the effect of the two steroids, the more powerful inhibitory effect of 5α-THB at 24 h compared with B suggests that 5α-THB may work predominantly by limiting the amount of PMNs, and therefore most likely neutrophils, recruited to the site of injury rather than influencing the onset of oedema. This would also be in accordance with the fact that the anti-inflammatory effect was seen at 24 but not at 6 h post-treatment.

GCs have been shown previously to inhibit migration of neutrophils (Pitzalis, Pipitone et al. 2002), and promote their phagocytosis by macrophages therefore stimulating the resolution of inflammation (Heasman, Giles et al. 2003). The work presented in this chapter was limited in that it analysed only one cell population. Future work needs to address the issue of cell infiltration in a quantitative manner by using techniques such as fluorescence-activated cell sorting (FACS). In this way, the numbers of cells infiltrating the ear tissue in response to croton oil and steroids could be investigated; more importantly, analysis of the different populations of cells present would also be possible.

Oedema and recruitment of inflammatory cells, including neutrophils, to the site of inflammation are processes that include many steps, from the release of inflammatory cytokines (i.e. IL1β) from keratinocytes and resident cells (i.e. Langerhans cells) to changes in vascular permeability and the expression of adhesion molecules (Grabbe and Schwarz 1998; Tsunemi, Saeki et al. 2006). To understand which of these steps may be influenced by 5α-THB and B, analysis of transcripts for a variety of genes was performed. Croton oil application significantly elevated

abundance of mRNAs of pro-inflammatory genes and of the anti-inflammatory gene *Dusp1*; B and 5α-THB, compared with the croton oil group, significantly reduced the abundance of transcripts of *Infγ* only, and increased that of *Annexin 1A*, whereas only 5α-THB increased significantly also that of *Dusp1*. Increase in the abundance of protein or transcripts of *Il6*, *Il1β* and *Tnfα* following croton oil application has been reported before (Towbin, Pignat et al. 1995; Ku, Jeong et al. 2009; Chen, Li et al. 2012; da Silva, Sperotto et al. 2013; Klose, Zigrino et al. 2013; Mo, Panichayupakaranant et al. 2013; Tian, Matsuo et al. 2013). INFγ is a pro-inflammatory factor mainly produced by Th1 cells, belonging to the adaptive immune system. It is known that the inflammatory cells found in irritant contact dermatitis are mainly cells of the innate immune response, as opposed to those found in allergic contact dermatitis. However, there is some evidence indicating that cells of the adaptive immune response may also play a role in irritant dermatitis (Kawase, Hoshino et al. 2003; Tsunemi, Saeki et al. 2006). The reduction in the amount of transcripts of *Infγ* seen with B and 5α-THB may indicate that they reduce the recruitment of lymphocytes to the site of inflammation. As mentioned above, the use of techniques such as FACS would help to clarify the cellular targets of the two steroids.

DUSP1, or dual specificity phosphatase 1, is an anti-inflammatory protein which, to our knowledge, has never been studied in models of irritant dermatitis, and therefore the findings presented here are new. Expression of DUSP1 is known to increase in response to a variety of inflammatory and irritant stimuli (Ryser, Massiha et al. 2004), such as dinitrofluorobenzene (DNFB) (Ayush, Lee et al. 2012), representing a negative feedback mechanism through which the inflammatory response is controlled. In effect, DUSP1 is a negative regulator of the MAPK signal transduction pathways, activation of which leads to production of cytokines, chemokines and adhesion molecules (Ayush, Lee et al. 2012). Its abundance is increased by conventional glucocorticoids (Kassel, Sancono et al. 2001), by non-steroidal anti-inflammatory compounds such as the non-essential amino acid l-glutamine (Ayush, Lee et al. 2012), and by new selective modulators of the glucocorticoid receptor (Joanny, Ding et al. 2012). The increase in transcripts abundance induced by 5α-

THB suggests that this may represent a mechanism through which this steroid works. Another anti-inflammatory gene tested was annexin A1, known also as lipocortin. The protein encoded by the gene was originally described as one of the molecules responsible for the anti-inflammatory properties of glucocorticoids (Oliani, Ciocca et al. 2008), and acts as a regulator of cells of the innate immune response. It has been shown that its expression on the surface of neutrophils inhibits their adhesion to endothelial cells diminishing their recruitment (Oliani, Ciocca et al. 2008). B and 5α-THB moderately increased abundance of transcripts of annexin A1 in inflamed ears indicating a possible molecular mechanism for the reduction in neutrophil infiltration seen with the two steroids.

When a panel of transcripts of genes controlling the vasculature and adhesion of inflammatory cells was investigated, B and 5α-THB were found to similarly decreased the abundance of those of *Vegfα*, *Pecam1*, *Icam1* and *Ve-cadherin* in inflamed ears. Disruption of the epidermal barrier leads to activation of endothelial cells (ECs) by factors such as VEGFα. This factor has been previously shown to be highly expressed in response to croton oil application (Bae, Shim et al. 2010). This protein is a potent activator of the vasculature, increasing the expression of permeability and adhesion molecule (i.e. ICAM1) and representing one of the first molecules to be produced locally after injury, most likely by keratinocytes (Bae, Shim et al. 2010; Zeng, Zheng et al. 2013). Many studies have directly investigated croton oil-induced increases in permeability by use of Evans blue (Shin, Joo et al. 2010; Chen, Li et al. 2012; Kobayashi, Tsubosaka et al. 2013; Sarashina, Tsubosaka et al. 2013), but only a few have analysed the process from a molecular point of view (Gahring, Osborne et al. 2010; Tian, Matsuo et al. 2013). Recruitment of inflammatory cells to the site of inflammation is a process involving different steps including tethering to and rolling on the endothelium, adhesion and paracellular migration (Hebeda, Teixeira et al. 2008). The process is coordinated by expression of adhesion molecules. E-selectin and ICAM1 are important in tethering, rolling and adhesion, while PECAM1 and VE-cadherin are involved in paracellular migration. It is know that glucocorticoids inhibit endothelium-leukocyte interaction (Cavalcanti, Lotufo et al. 2007; Hebeda, Teixeira et al. 2008). B and 5α-THB seem to modulate

each of the steps, and they could do so either by affecting the transcription of these molecules, via for example inhibition of the NF-κB pathway, which is known to be affected by steroids in endothelial cells (Brostjan, Anrather et al. 1996), or by inhibiting the expression of other permeability-promoting protein such as VEGFα.

Effective recruitment of inflammatory cells and tissue repair after injury are mechanisms dependent on tissue remodelling. Important molecules involved in this process belong to the family of metalloproteinases (MMPs); they cleave different kinds of extracellular matrix components, freeing space for cells to move and tissue to be re-built. Furthermore, complete recovery of the skin structure after injury is only possible if there is deposition of extracellular structural proteins, such as collagen. Disruption of the epidermal barrier by croton oil is a traumatic event that leads to activation and death of mainly keratinocytes. The combined action of MMPs, structural protein such as collagen 4a1, which forms the basement membrane beneath the epidermal and endothelial layers, and cytoskeletal proteins that allow proliferation and cellular migration, represents the response to the injury through which the skin heals itself. This is well represented in this chapter's model by the increase induced by croton oil of the abundance of transcripts of genes involved in tissue remodelling. Both 5α-THB and B similarly decreased that of *Col4a1* and *Actin* in inflamed ears, while only B decreased that of *Mmp9* and *Keratin6*. It is worth noting that the effect of 5α-THB on *Mmp9* and *Keratin6*, despite not being significantly different from the croton oil group, is not different from the group treated with B either. This indicates that the results of the statistical test have probably been influenced by a larger variability in the 5α-THB group, and that also this steroid may be decreasing transcription of the two genes.

Inhibition of transcription by GCs has been reported in the literature for all the genes mentioned, although mainly under non-inflammatory conditions and mostly in *in vitro* experiments targeted to specific cell types (keratinocytes and endothelial cells) (Dhabhar and McEwen 1999; Radoja, Komine et al. 2000; Stojadinovic, Lee et al. 2007; Donet, Bosch et al. 2008; Shikatani, Trifonova et al. 2012). The protein keratin6 is a marker of activated keratinocytes and hyper-proliferative epidermis and is known to be repressed by glucocorticoids (Radoja, Komine et al. 2000;

Stojadinovic, Lee et al. 2007; Donet, Bosch et al. 2008), while actin is a cytoskeletal component universally present in cells. These factors, together with collagen, are important in tissue remodelling/scar formation which are processes involving cell proliferation (Stojadinovic, Lee et al. 2007). It is known that steroids cause skin thinning, and this side effect is likely due to their inhibitory action on MMPs, cell proliferation (i.e. of keratinocytes) and structural proteins (Stojadinovic, Lee et al. 2007). The data presented here though do not necessarily suggest a negative impact on the healing process and skin thickness. Indeed, a likely possibility is that the decrease in abundance of the transcripts of these genes is an indirect effect of the reduction of inflammation caused by the two steroids. Previous unpublished data showed that 5α-THB, in contrast to B, did not inhibit transcription of collagen genes, and did not cause skin thinning when applied to uninjured and non-inflamed skin. To better clarify the effects of this compound on structural molecules with an important role in maintaining skin integrity, wider approaches such as microarray or RNAseq analysis would be useful.

It is worth taking into consideration that the results obtained from the study of abundance of transcripts may not reflect the abundance of protein in the tissue. GCs have been shown to inhibit translation rather than transcription for some molecules involved in inflammation (Clark 2003; Clark, Dean et al. 2003). As a consequence, while no significant effect of 5α-THB and B was found on transcription of cytokines, it may well be possible that they may be affecting the translation of those transcripts. By the same token, any significant effect seen regarding adhesion molecules, vascular and remodelling factors should be further verified by analysis of protein abundance, either by ELISA, Western blotting or immunohistochemistry.

In order to investigate the involvement of the glucocorticoid receptor in the anti-inflammatory properties shown by 5α-THB and B, the literature was reviewed for previous studies using the GR receptor antagonist RU486 in the croton oil model of irritant dermatitis. A small number of papers was retrieved (Iwasaki, Mishima et al. 1995; Pinto, Morais et al. 2010) and adaptation of their methods was carried out in order to efficiently deliver the drug. The lack of an effect of topical application of

RU486 on the anti-inflammatory properties of B suggested that his method was not efficiently delivering the drug to GR in the skin. Previous work in literature (Iwasaki, Mishima et al. 1995) used creams containing RU486, and this indicates a possible issue with delivery from the vehicle used in the experiment presented here. It was also possible that the concurrent administration of RU486 and B has played a role, since routinely the antagonist is given before the agonist. The results obtained with subcutaneous injections of RU486 showed that the antagonist could partially relieve the action of B; however, injection of the vehicle DMSO also had anti-inflammatory effects that could be partially reversed by RU486. There is the possibility that DMSO may have had anti-inflammatory properties *per se*, as shown previously in this model (Coruzzi, Pozzoli et al. 2011); another plausible scenario is that a glucocorticoid-dependent stress response was taking place as suggested by the effect of RU486 in mice injected with DMSO. It is worth noting that control groups were never included in the previous publications mentioned above, leaving one wondering about the true interpretation of the data published.

In order to resolve the issue described above, two measures were adopted simultaneously: removal of the adrenal glands and change of the vehicle in which RU486 was dissolved from DMSO to ethanol. It could be argued that the best approach would have been to test first whether DMSO represented the main problem, and then, based on the results, proceed with the adrenalectomy. However, based on the experience of my supervisors' group with the high variability of this model, applying the two measures together was judged as the best approach to tackle two possible issues while at the same time sparing important resources.

From the experiments a surprising result emerged: while RU486 relieved the anti-inflammatory effect of B this was not the case for 5α-THB. This finding was unexpected because 5α-THB has been shown to displace dexamethasone from rat GR (McInnes, Kenyon et al. 2004). However, 5α-THB has never been tested *in vivo* in the presence of RU486 before, and these results indicate that binding to GR may not occur in this context in which many molecular and cellular players are involved, in contrast to the *in vitro* scenario. A novel selective glucocorticoid receptor

modulator, called Compound A, has shown a promising dissociated profile in different *in vitro* and *in vivo* models. However, some aspects of its action are not GR-dependent (Beck, Drebert et al. 2013). In line with this, 5α-THB's behaviour may not be totally dependent on GR either, which may also explain the different time-course, compared to B, of its anti-inflammatory effects in the dermatitis model.

The fact that 5α-THB, naturally produced in the body, shows anti-inflammatory properties, raises the intriguing possibility that it may have a role in the physiological response to inflammatory stimuli. Two enzymes are responsible for its production, 5α-R1 and 5α-R2, and only transcripts for 5α-R1 were detected in skin by PCR. Previous research has shown the expression of both in genital and non-genital skin (Nixon, Upreti et al. 2011), in human and rat samples. This is the first study, to our knowledge, that analysed the transcription of these enzymes in murine skin. Quantitative analysis of transcripts was also performed, and their abundance was decreased by treatment with croton oil. This pattern suggests a local regulation of the action of glucocorticoids and their metabolism by croton oil, or by a component of the response to croton oil which is not altered by GCs. This is possibly a mechanism put in place by cells in order to re-establish tissue homeostasis after injury by reducing metabolism of endogenous GCs and, therefore, promoting the anti-inflammatory action of the substrate steroid. However, the data may also reflect a decrease in the proportion of 5α-R1 expressing cells as a new inflammatory cell population not transcribing the gene may infiltrate the ears. Indeed, the PCR analysis presented here was performed in non-inflamed skin, containing virtually no cells of the circulating immune system; since a reduction in the amount of transcripts of *Srd5a1* was seen after croton oil application, it seems unlikely that infiltrating immune cells express the gene. In humans and rats, 5α-R1 seems more widely distributed than 5α-R2 in the immune system, but previous work in mice showed no transcripts in murine bone marrow-derive macrophages (Yang, Nixon et al. 2011).

In physiological conditions, the HPA axis maintains the concentration of GCs in the circulation within certain values. Emotional or physical stress triggers the release of corticotrophin-releasing hormone (CRH) from the hypothalamus, which in turn

stimulates the production of adrenocorticotropic hormone (ACTH) from the anterior pituitary; this drives the release of cortisol (in humans) or B (in rodents) from the adrenal gland into the blood stream (Keller-Wood and Dallman 1984). This pathway is controlled by negative feedbacks in order to return the concentration of cortisol or B to basal levels once the stressful stimulus is no longer present. The concentration of B in plasma in wild type and 5α-R1$^{-/-}$ mice at cull, after treatment with croton oil, was higher than that measured in unstressed mice in previous studies (Livingstone, Di Rollo et al. 2014), but it was comparable to that measured under mild stress (handling stress). This suggests that the animals were stressed, probably because of cage disturbance, at the moment of cull. No differences were found between genotypes in the studies presented in this chapter, in contrast to the investigation mentioned above. This may have been due to differences between the two studies in the duration of the stressful stimulus, and in the way the stress was induced. It would be of interest to quantify plasma B in response to the handling stress induced by topical treatment. However, because the effects of croton oil and steroids were comparable in the two genotypes, any difference concerning B concentration in response to stress is unlikely to have played a role in this model.

To test for a putative role of 5α-R1 in inflammation and response to steroidal treatment, mice with genetic disruption of 5α-R1 (5α-R1$^{-/-}$) were employed. The results obtained indicate that local metabolism of glucocorticoids in the ear tissue does not play an important role in either the physiological inflammatory response or in the anti-inflammatory properties of 5α-THB or B. As mentioned above, previous studies (Livingstone, Barat et al. 2014) showed that 5α-R1$^{-/-}$ mice had a worse hepatic metabolic phenotype than wild type littermates, suggesting an important role for the production of 5α-THB in controlling local physiology. Other reports showed that local metabolism is a central factor in determining the local availability of GCs and their effects, both on physiology and pathology (Tiganescu, Walker et al. 2011; Yau and Seckl 2012; Morgan, McCabe et al. 2014; Terao, Tani et al. 2014; Tiganescu, Hupe et al. 2014). The present study did not show a role of local A-ring metabolism of GCs in the response to irritation of the skin, suggesting perhaps that the physiological amount of GCs or/and the activity of the enzyme in adult mice skin

may be too low to be of any influence. A remark worth making is that even though I showed the presence of transcripts of *Srd5a1* in ears, this may not correlate with the presence of a functional enzyme.

In summary, 5α-THB is anti-inflammatory, albeit in a less potent fashion than B, and shows a different time course of efficacy compared with the compound from which it derives. Investigation of the molecular mechanisms revealed that the two steroids influenced anti-inflammatory pathways in a similar manner. However, a couple of exciting differences were found. Firstly, 5α-THB significantly increased abundance of the transcripts of *Dusp1*, a central anti-inflammatory gene. This result confirmed unpublished findings (Dr. Alastair Jubb, personal communication) which showed *Dusp1* transcripts to be increased by 5α-THB in human monocyte-derived macrophages. Secondly, the anti-inflammatory properties of 5α-THB, in contrast to B, were not relieved by the GR antagonist RU486. This finding suggests that the reason behind the lack of side effects seen with the use of 5α-THB may lay upstream of any molecular pathways investigated, specifically on the receptor to which it is bound. This is an exciting discovery that, while challenging the current knowledge about how GCs work, also opens new doors to a better scientific understanding of how they work *in vivo*, and offers new opportunities for research into alternative anti-inflammatory compounds.

Chapter 4

ANTI-INFLAMMATORY

PROPERTIES OF 5α-THB *IN VITRO*

Chapter 4: Anti-inflammatory properties of 5α-THB *in vitro*

4.1 Introduction

In Chapter 3, 5α-THB was shown to possess anti-inflammatory properties in an *in vivo* model of inflammation. Surprisingly, these effects were not antagonised by the GR antagonist RU486. In order to complement these data, the ability of this 5α-reduced steroid to suppress the release of cytokines from macrophages in a GR-dependent manner was investigated in simpler cellular *in vitro* models.

Classically, macrophages in culture are activated by cytokines such as TNFα, or molecules such as lipopolysaccharide (LPS), a major component of the membrane of gram-negative bacteria. LPS stimulates macrophages by binding to the Toll-like receptor (TLR) 4, and triggering the activation of intracellular signalling pathways which leads to the production of pro-inflammatory cytokines (i.e. IL6, TNFα and IL1β) through the action of transcription factors such as NF-κB and AP-1 (Nissen and Yamamoto 2000; Kracht and Saklatvala 2002; De Bosscher, Vanden Berghe et al. 2003; Fukao and Koyasu 2003; Fang, Pengal et al. 2004; Hayden and Ghosh 2008; O'Neill 2008; O'Neill 2008). Despite monumental efforts to determine how GCs work to inhibit inflammation, the mechanisms involved are not yet fully understood. There is strong evidence, though, that the binding of GR monomers to NF-κB and AP-1, with consequent blockage of their transcriptional activity, is one of the principal mechanisms involved (Schoneveld, Gaemers et al. 2004; Barnes 2006; De Bosscher and Haegeman 2009). This mechanism is referred to as trans-repression. GCs also interfere with pro-inflammatory stimuli in a more indirect manner. For instance, they are known to suppress the activation of p38 MAPK by inducing the expression of the protein MAPK phosphatase 1 (MKP1), also known as DUSP1, and annexin 1 by a mechanism that is classically called trans-activation and is dependent mainly, but not only, on binding to DNA of GR homodimers (Barnes 2006; Bhattacharyya, Brown et al. 2007; Ayroldi, Cannarile et al. 2012; Vandevyver, Dejager et al. 2012; Vandevyver, Dejager et al. 2013).

To shed light on the molecular mechanisms potentially involved in the anti-inflammatory properties of 5α-THB, the cell line RAW264.7 and murine bone marrow-derived macrophages were employed in the studies presented in this chapter.

Hypothesis

5α-THB suppresses the release of pro-inflammatory cytokines from stimulated macrophages in culture through actions mediated by GR.

Objective

The objective of this chapter is:

- To investigate the anti-inflammatory properties of 5α-THB in comparison with B, in cell culture models of inflammation by biochemical and molecular analysis.

- To assess if the anti-inflammatory effects of 5α-THB are antagonised by the GR antagonist RU486.

4.2 Material and methods

4.2.1 General

4.2.1.1 Materials

Materials for general cell culture and experimental treatments were obtained as described in section 2.1.1.1. Steroids, including the GR antagonist RU486, were from Steraloids (Newport, RI, USA); lipopolysaccharide (LPS) was from Sigma-Aldrich (Dorset, UK).

4.2.1.2 Preparation of steroids and LPS

Steroids were dissolved in ethanol, and LPS diluted in sterile PBS, to obtain solutions 1000 times more concentrated than required. This allowed the addition of 1 μL of steroid or LPS solutions into each mL of medium at the time of treatment, minimising the effect of ethanolic vehicle. The solutions containing steroids were stored at -20 ºC for a maximum of one month before being replaced. LPS solutions were stored at 4 ºC. When needed, the solutions were allowed to stand at RT for 30 min prior to use.

4.2.1.3 General treatments

Stimulation of macrophages was performed by treatment with LPS at the concentration and for the time indicated. A "vehicle group" was always included by treating the cells with PBS instead of LPS. When steroidal treatments were used, the cells were pre-incubated for 1 h with steroids or vehicle (ethanol) prior to stimulation with LPS. Details are given about specific adjustments made throughout the experiments. Each treatment was performed in triplicate, and each experiment repeated between three and eight times, as indicated.

4.2.1.4 ELISA

ELISA was performed as described in section 2.3.3.3.

4.2.2 RAW264.7 cells

4.2.2.1 Source of cells

The cell line RAW264.7 was obtained as described in section 2.1.1.2.1.

4.2.2.2 Cell culture

Cells were thawed as described in section 2.2.2. They were maintained in culture in T75 flasks in normal-serum medium (prepared as described in section 2.1.1.3.2), and passaged as described in section 2.1.2 When a confluence of approximately 80% was reached, they were passaged into 12-well culture plates at a concentration of 3-4 x 10^5 cells/well. Twenty four hours later, they were washed twice with PBS and the medium was replaced with stripped-serum medium, prepared as described in section 2.1.1.3.2. After 24 h, the experimental treatments were started.

4.2.2.3 Adjustment of the treatments

Development of an *in vitro* model of inflammation

To select the time at which to analyse the anti-inflammatory properties of 5α-THB compared with B, RAW264.7 cells were pre-treated with the positive control B (1 μM) and stimulated with LPS for 4 and 24 h. The amount of LPS to use (30 ng/mL) was calculated in previous experiments performed in my supervisors' group (Nixon 2011) and represented a sub-maximal concentration of LPS.

Optimization of the anti-inflammatory response with 5α-THB

The effect of 5α-THB on the abundance of transcripts for cytokines was firstly investigated employing an equivalent concentration to B (1 μM). Thereafter, to assess if the response obtained with the compound was maximal, cells were pre-treated with steroid at a range of concentrations (1-10 μM) before being stimulated with LPS for 24 h.

Time-dependent variations of levels of pro-inflammatory cytokines

To assess the time-course of response of pro-inflammatory cytokines, cells were stimulated with LPS for 1, 2, 4, 8, 16 and 24 h in the presence or absence of steroids, and transcripts for IL6 and TNFα were quantified by real-time PCR as described in section 2.3.1.6. Production of IL6 after stimulation either with LPS alone or LPS and steroids for 4, 8, 16 and 24 h was performed using ELISA.

Investigation of the causes for the lack of reproducibility

The results obtained showed that the *in vitro* model of inflammation based on the use of LPS-stimulated RAW264.7 cells was not reproducible with respect to the data obtained through real-time PCR and ELISA regarding pro-inflammatory cytokines. The following steps were taken to address this problem:

1) comparison of different batches of 5α-THB prepared at different times prior to the beginning of experiments
2) utilisation of a new batch of RAW264.7 cells, purchased afresh

The following outcomes were assessed: the abundance of transcripts for pro-inflammatory cytokines was measured after 24 h treatment either with LPS or LPS plus steroids, using 5α-THB at 1, 3 and 10 μM. Production of cytokines was measured after treatment for 4, 8, 16 and 24 h using ELISA.

4.2.2.4 Real-time PCR

Total RNA was extracted and mRNA abundance quantified as described in sections 2.3.1.2 and 2.3.1.6. The primers employed were as described in Table 2.1, Chapter 2.

4.2.2.4.1 Data analysis of real-time PCR data

For quantification of transcripts through real-time PCR, the abundance of mRNAs for each gene was represented in relation to the abundance of a chosen housekeeping gene. The housekeeping gene used was *Tbp* due to the lack of significant changes in the abundance of transcripts following treatment with LPS or LPS + steroids. For each sample and each gene studied the values presented in the graphs were calculated as follow: value sample X/value *Tbp* sample X.

4.2.3 Murine bone marrow-derived macrophages

4.2.3.1 Source of cells and materials

Bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6 mice as described in section 2.1.1.2.2.

4.2.3.2 Culture

BMDMs were cultured and allowed to differentiate as described in section 2.1.1.2.2. On the 6th day of culture, normal-serum medium was replaced with serum-stripped medium (both prepared as described in section 2.1.1.3) after two washes with PBS.

After 24 h, treatments either with LPS alone or in conjunction with steroids were started.

4.2.3.3 Treatments

Steroidal solutions were prepared as described in section 4.2.1.2. In addition, the GR antagonist RU486 was used. The compound was dissolved in ethanol prior to each experiment, at a concentration 1000 times higher than the final concentration in the culture medium.

Treatments either with LPS alone or B or 5α-THB + LPS were performed as described in section 4.2.1.3. Treatments with RU486 (or vehicle) were started one hour prior to treatment with steroids (or vehicle in the case of the LPS only-treated group), followed by treatment with LPS.

4.2.3.4 Western blotting

4.2.3.4.1 Materials

General materials were obtained as described in section 2.3.3.1.1. Specific primary antibodies employed were: monoclonal rabbit anti-NF-κB and monoclonal rabbit anti-IκBα from New England Biolabs, Ltd (Hitchin, Hertfordshire, UK); monoclonal mouse anti-β tubulin and monoclonal mouse anti-GAPDH from Insight Biotechnology (Wembley, Middlesex, UK). Secondary antibodies were: IRDye 800CW Goat Anti-Rabbit IgG (H+L) and IRDye 800CW Goat Anti-mouse IgG (H+L) from LI-COR Biosciences Ltd (Cambridge, Cambridgeshire, UK). Protein extraction from BMDMs, quantification and Western blotting analysis were performed as described in sections 2.3.3.1.2, 2.2.3.1.3 and 2.3.3.1.4. All primary antibodies were diluted 1:1000 in BSA (5% w/v in TBST), and all secondary antibodies were diluted 1:10000 in Milk (5% w/v in TBST).

4.2.3.4.2 Procedure

Protein extraction from BMDMs, quantification and Western blotting analysis were performed as described in sections 2.3.3.1.2, 2.2.3.1.3 and 2.3.3.1.4.

4.2.3.4.3 Quantification of protein expression

The abundance of protein was calculated by using the Odyssey® Imaging software (LI-COR biosciences, Cambridge, Cambridgeshire, UK). In order to normalise the abundance of the proteins of interest to the total amount of proteins present in the blots, the intensity values associated with the bands representing NF-κB and IκBα were divided by the values of the corresponding bands representing GAPDH and β-Tubulin respectively, and graphed as such. Two different proteins were chosen as loading controls in order to avoid interfering signals from the antibodies; this choice made it possible to detect the protein of interest and either GAPDH or β-Tubulin on the same blot, without the necessity for stripping and re-probing.

4.2.4 Data analysis

Data were represented as mean ± SEM, and analysed as described in section 2.4.

4.3 Results

4.3.1 Development of a glucocorticoid sensitive-model of inflammation in RAW264.7 cells

At both 4 and 24 h post LPS stimulation, the abundance of mRNAs of the genes *Il6* and *Tnfα* in the LPS-treated group was higher compared with the vehicle-treated cells (Figure 4.1 a, b); for *Il6* this increase was more pronounced at 24 h than at 6 h, while for Tnfα was comparable at both time points. Treatment with B reduced the amount of transcripts at both end points (Figure 4.1 a) compared with LPS-treated cells, having a bigger effect at 24h. Following these observations it was decided to stimulate the cells with LPS for a period of 24 h.

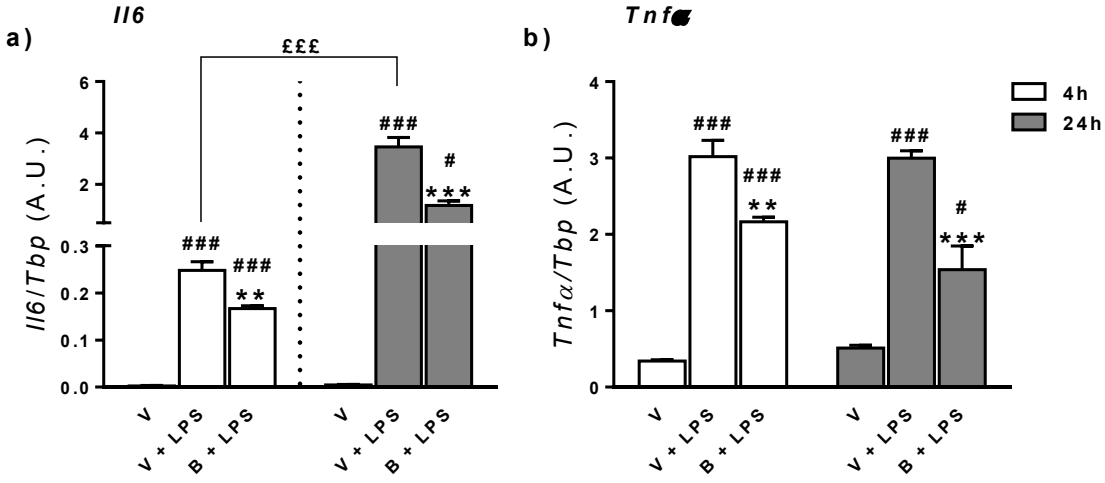

Figure 4.1 Treatment with lipopolysaccharide (LPS) for 4 and 24 hours (h) increased the abundance of transcripts of the genes *Il6* and *Tnfα* in RAW264.7 cells; this increase was attenuated by corticosterone (B). Real-time PCR analysis of the abundance of transcripts of the pro-inflammatory genes (a) *Il6* and (b) *Tnfα* after treatment for 4 or 24 h with either vehicle (V), LPS (30 ng/mL) alone or B (1 μM) + LPS. Data (mean ± SEM) were analysed by one-way ANOVA with Tukey's post-hoc test; ### = $p < 0.0001$, # $p < 0.05$ vs matched V; *** = $p < 0.0001$, ** = $p < 0.001$ vs matched V + LPS; £££ = $p < 0.0001$; N = 4. *Tbp* = TATA-box binding protein, A.U. = arbitrary unit.

4.3.2 Ability of 5α-THB to suppress the inflammatory response in RAW264.7 cells

4.3.2.1 Response of the model to 5α-THB

In the initial experiments, treatment for 24 h with B and 5α-THB (1 μM) reduced the abundance of transcripts of *Il6*, *Tnfα* and *Il1β* compared with LPS only treated cells (Figure 4.2 a-c). In all cases, B had a greater effect than 5α-THB. The steroids reduced to a similar extent also the basal amount (non-stimulated cells) of transcripts of *Tnfα* and *Il1β* (Figure 4.2 b and c, white columns).

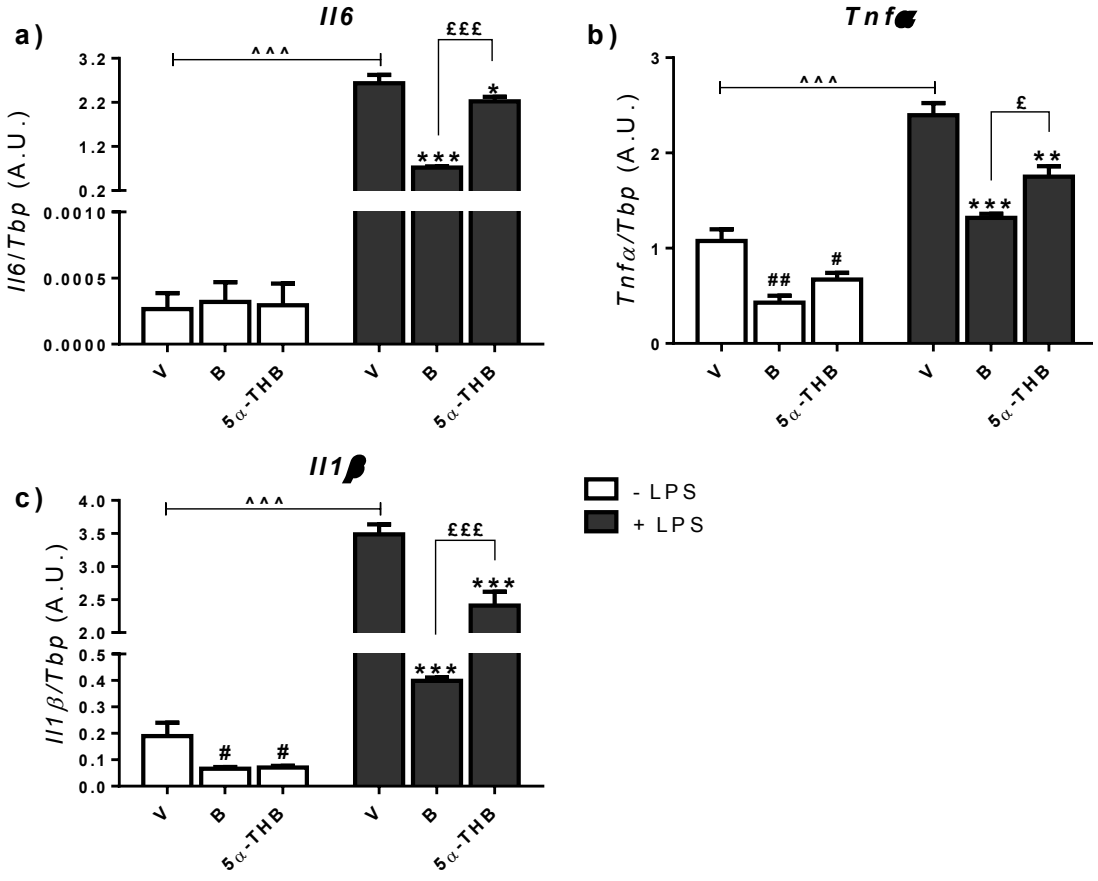

Figure 4.2 5α-Tetrahydrocorticosterone (5α-THB) lowered the abundance of mRNAs of *Il6*, *Tnfα* and *Il1β* following stimulation with lipopolysaccharide (LPS). Real-time PCR analysis of the abundance of transcripts of the genes (a) *Il6*, (b) *Tnfα* and (c) *Il1β* following treatment with either vehicle (V), corticosterone (B) or 5α-THB (both 1μM), either in presence or absence of LPS (30 ng/mL). Data (mean ± SEM) were analysed by one-way ANOVA with Tukey's post-hoc test; ^^^ = $p < 0.0001$; ## = $p < 0.001$, # $p < 0.05$ vs matched V; *** = $p < 0.0001$, ** = $p < 0.001$ vs matched V; £££ = $p < 0.0001$, £ = $p < 0.05$; N = 6. *Tbp* = TATA-box binding protein, A.U. = arbitrary unit.

4.3.2.2 Concentration-dependent response of glucocorticoids to decrease transcripts of inflammatory genes

As above, stimulation with LPS for 24 h increased the abundance of transcripts of *Il6*, *Tnfα* and *Il1β* in comparison with unstimulated cells (Figure 4.3 a-c); treatment with B decreased the basal amount of transcripts of all genes, while treatment with 5α-THB decreased only that of *Il1β* (Figure 4.3 a-c white columns). Pre-treatment with B (1 and 3 μM) decreased the abundance of mRNAs of all genes in cells stimulated with LPS; 5α-THB reduced the amount of transcripts in stimulated cells only when given at 3 μM, and the effect was comparable with that of the same concentration of B only for *Tnfα* (Figure 4.3 a-c black columns).

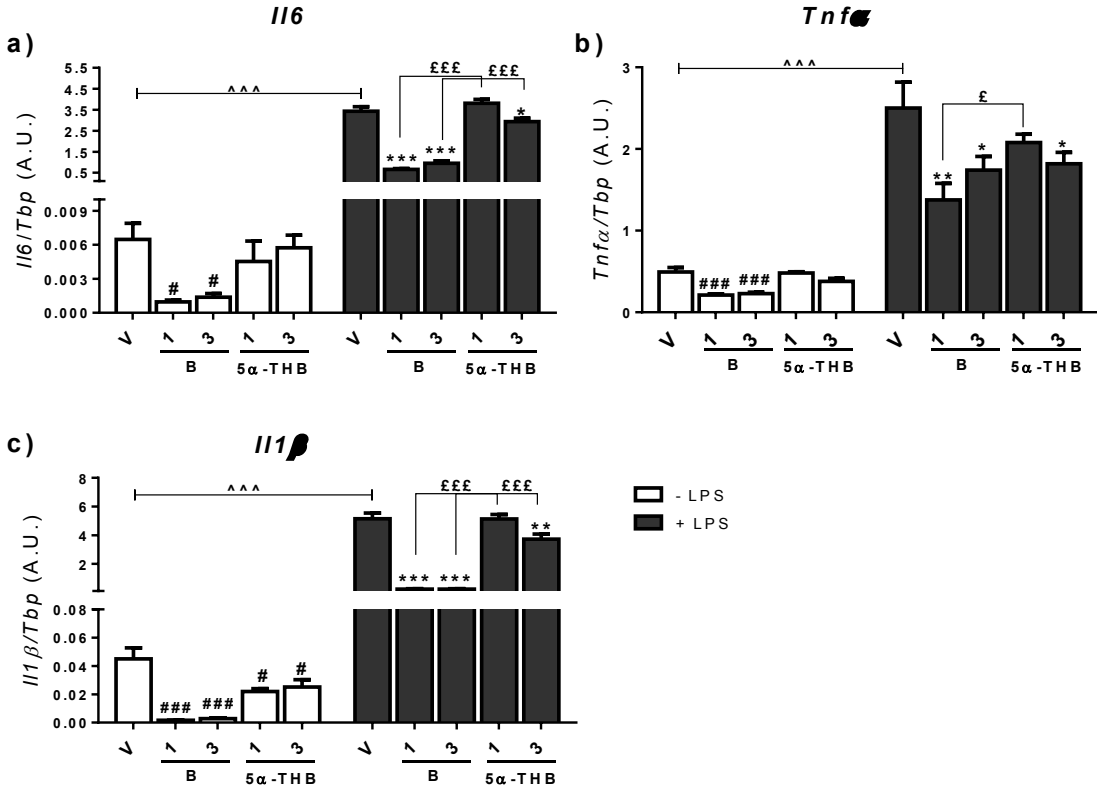

Figure 4.3 5α-Tetrahydrocorticosterone (5α-THB) decreased lipopolysaccharide (LPS)-induced increase of transcripts of inflammatory genes when given at 3 but not at 1 μM. Quantification by real-time PCR of the abundance of mRNAs of (a) *Il6*, (b) *Tnfα* and (c) *Il1β* following treatment (24 h) with either vehicle (V), corticosterone (B) or 5α-THB either in the presence or absence of LPS (30 ng/mL) stimulation. Numbers after steroids indicate the concentration in μM. Data (mean ± SEM) were analysed by one-way ANOVA with Tukey's post-hoc test. ^^^ and £££ = $p < 0.0001$, £ = $p < 0.05$; ### = $p<0.001$; # = $p< 0.05$ vs matched V; *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ vs matched V; N = 6. *Tbp* = TATA-box binding protein, A.U. = arbitrary unit.

4.3.3 Investigation of the possible causes for the lack of reproducibility

4.3.3.1 Time-dependent variation in the abundance of transcripts of inflammatory genes in response to 5α-THB and B

Given the conflicting data regarding the extent of the response with 5α-THB (compare Figure 4.3 with Figure 4.2), a time course of the abundance of mRNAs of *Il6*, *Tnfα* and *Il1β* following LPS stimulation, with or without pre-treatment with steroids, was conducted. Compared with cells treated with vehicle, treatment with LPS increased the abundance of mRNAs of *Il6* after 8 and 24 h; of *Tnfα* after 1, 2, 4, 8 and 24 h and of *Il1β* after 4, 8 and 24 h (Figure 4.4 a-c, squares vs circles). B (1 μM) reduced this increase after treatment lasting 24 h for *Il6*; 1 and 2 h for *Tnfα* and 4, 8 and 24 h for *Il1β*; in addition, B unexpectedly increased transcripts of *Il6* and *Tnfα* at 16 h (Figure 4.4 a-c, triangles). 5α-THB (1 μM) only reduced the increase caused by LPS of *Tnfα* after 1 h treatment and, like B, increased the abundance of transcripts of all genes at 16 h (Figure 4.4 a-c, inverted triangles).

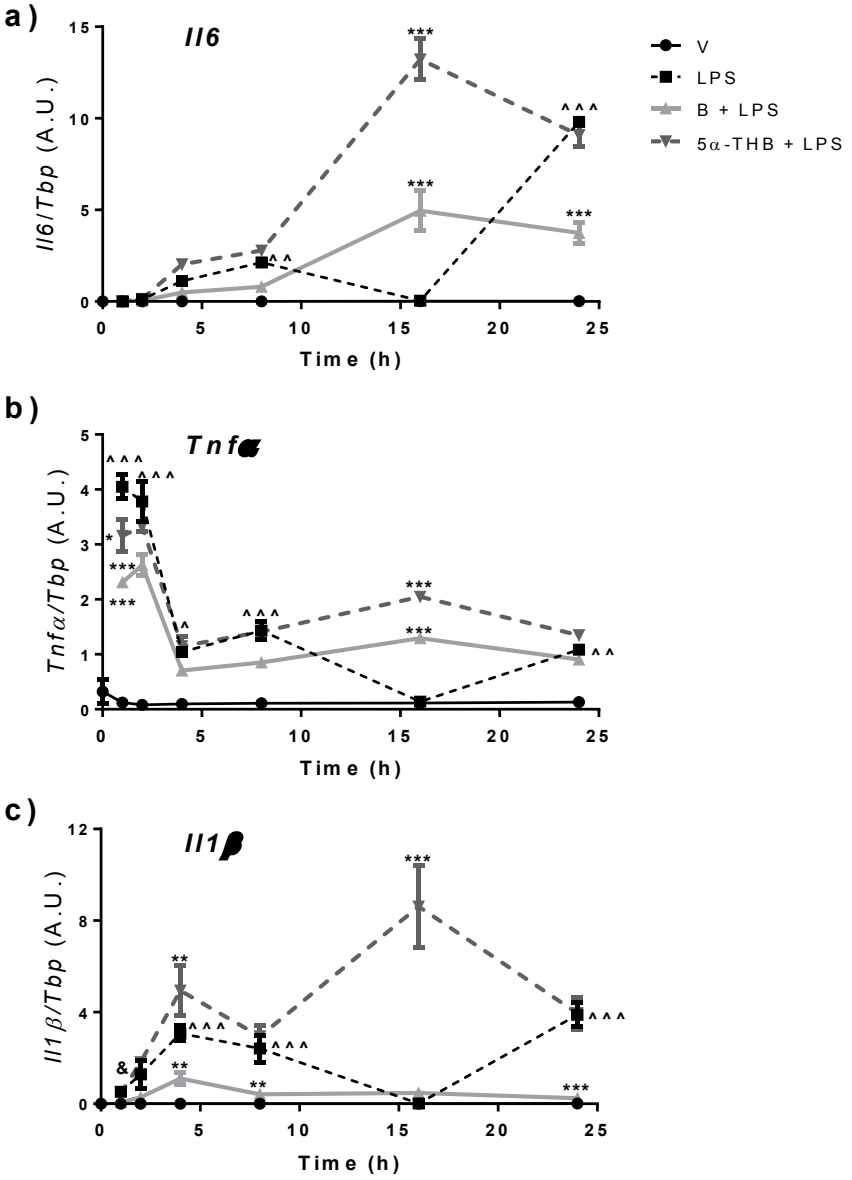

Figure 4.4 5α-Tetrahydrocorticosterone (5α-THB) did not affect the abundance of transcripts of pro-inflammatory genes at any time point analysed. Quantification by real-time PCR of the time-dependent changes in the abundance of transcripts of (a) *Il6*, (b) *Tnfα* and (c) *Il1β* following treatment with either vehicle (V), corticosterone (B) or 5α-THB (both 1 μM) of RAW264.7 cells, either in presence or absence of stimulation with lipopolysaccharide (LPS, 30 ng/mL). Data (mean ± SEM) were analysed with repeated measured ANOVA with Tukey's post-hoc test. *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ (steroid) vs (V + LPS); ^^^ = $p < 0.0001$, ^^ = $p < 0.001$, ^ = $p < 0.05$, & = $0.1 < p < 0.05$ (V + LPS) vs (V); N = 4. *Tbp* = TATA-box binding protein, A.U. = arbitrary unit.

4.3.3.2 Influence of 5α-THB solutions

Solutions of 5α-THB stored for different times prior to the execution of the experiments were analysed for their effects on the abundance of mRNAs of gene encoding cytokines after treatment for 24 h.

None of the 5α-THB solutions decreased the abundance of transcripts compared with the LPS-stimulated group (Figure 4.5 a, b); the use of 5α-THB solutions prepared 1 month prior to the experiments (A in the graphs) increased transcripts of *Tnfα* when given at 1 μM (Figure 4.5 b). The effects induced by the 1 month-old solution were significantly different from those of the solutions prepared 2 weeks prior to the experiments (B in the graphs), and the same day (C in the graphs); there was some variation according to the gene investigated (Figure 4.5 a, b). For this reason, it was decided that for future experiments the solutions of 5α-THB were to be freshly prepared each time and, for consistency, this approach was taken also for the solutions containing B.

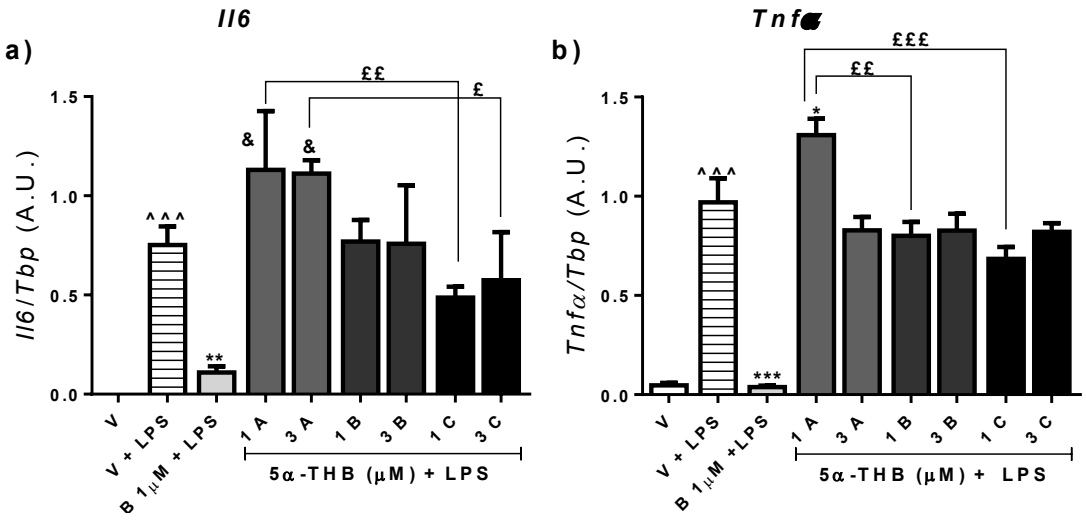

Figure 4.5 Solutions of 5α-tetrahydrocorticosterone (5α-THB) stored for different times differently affected the abundance of transcripts for cytokines. Quantification by real-time PCR of the abundance of transcripts for (a) IL6 and (b) TNFα following treatment with either vehicle (V), LPS alone (30 ng/mL) or lipopolysaccharide (LPS) + corticosterone (B) or 5α-THB for 24 h. A = 1 month-old solution, B = 2 week-old solution, C = solution prepared the same day. Data (mean ± SEM) were analysed with one-way ANOVA with Tukey's post-hoc test. ^^^ = $p < 0.0001$ vs V; *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$, & = $0.1 < p < 0.05$ vs V + LPS; £££ = $p < 0.0001$, ££ = $p < 0.001$, £ = $p < 0.05$; N = 4. Tbp = TATA-box binding protein, A.U. = arbitrary unit.

4.3.3.3 Investigation of a new batch of RAW264.7 cells

4.3.3.3.1 Concentration-dependent production of cytokines in response to LPS

To assess whether the variability in the response to 5α-THB was due to variations in cell performance following multiple passages, IL6 and TNFα production was quantified after stimulation with different concentrations of LPS for 24 h in fresh cells. As shown in Figure 4.6, the production of both cytokines by the new batch of cells increased in a concentration-dependent manner. As the concentration used in experiments performed previously (30 ng/mL, arrow in graph) was not causing a maximal response, it was decided to continue the investigation on the new batch of cells with the same concentration.

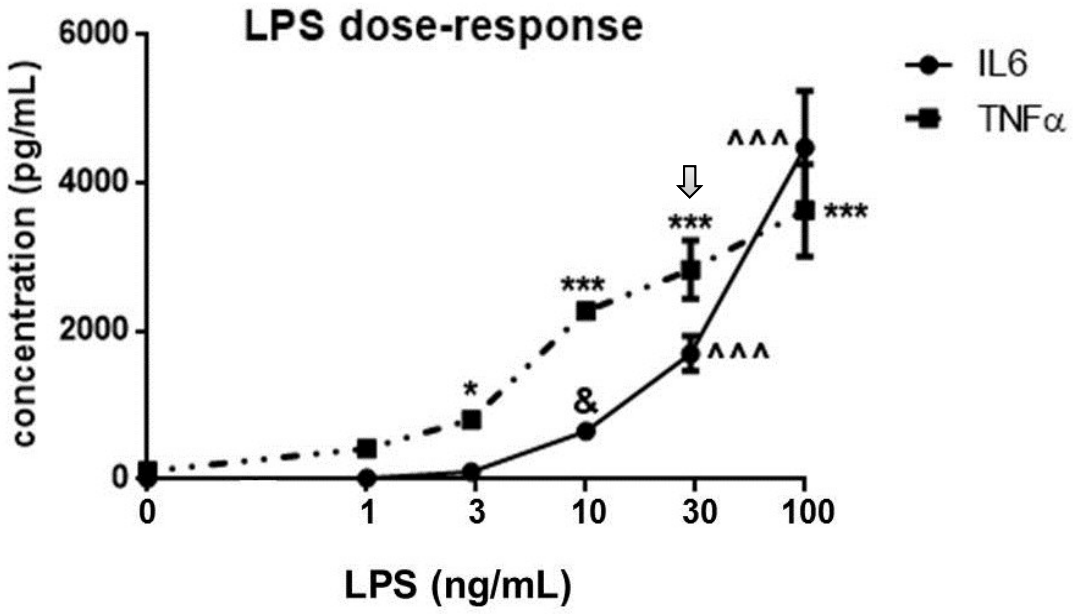

Figure 4.6 Lipopolysaccharide (LPS) stimulated the production of IL6 and TNFα in a concentration-dependent manner in a new batch of RAW264.7 cells. Cells were stimulated for 24 hours with increasing concentrations of LPS and the amounts of IL6 and TNFα quantified by ELISA. Data (mean ± SEM) were analysed with one-way ANOVA with Tukey's post-hoc test. *** and ^^^ = $p < 0.0001$, * = $p < 0.05$, & = $0.1 < p < 0.05$ vs vehicle (0 in the graph); N = 4. Arrow points to the concentration utilised in the experiments (30 ng/mL). The graph was drawn as a semi-log plot; the log values were substituted with the real concentrations of LPS to give a clearer representation.

4.3.3.3.2 Abundance of transcripts in response to steroids

The abundance of transcripts of *Il6*, *Tnfα* and *Il1β* increased in LPS-stimulated cells after 24 h of treatment compared with vehicle-treated group. B (1 μM) lowered this increase for each gene compared with LPS-treated cells; 5α-THB reduced the abundance of transcripts of *Il6* at all concentrations tested, but did so for *Il1β* only at 10 μM; in addition, at 3 μM it increased the abundance of mRNAs of *Tnfα* in stimulated cells (Figure 4.7 a-c).

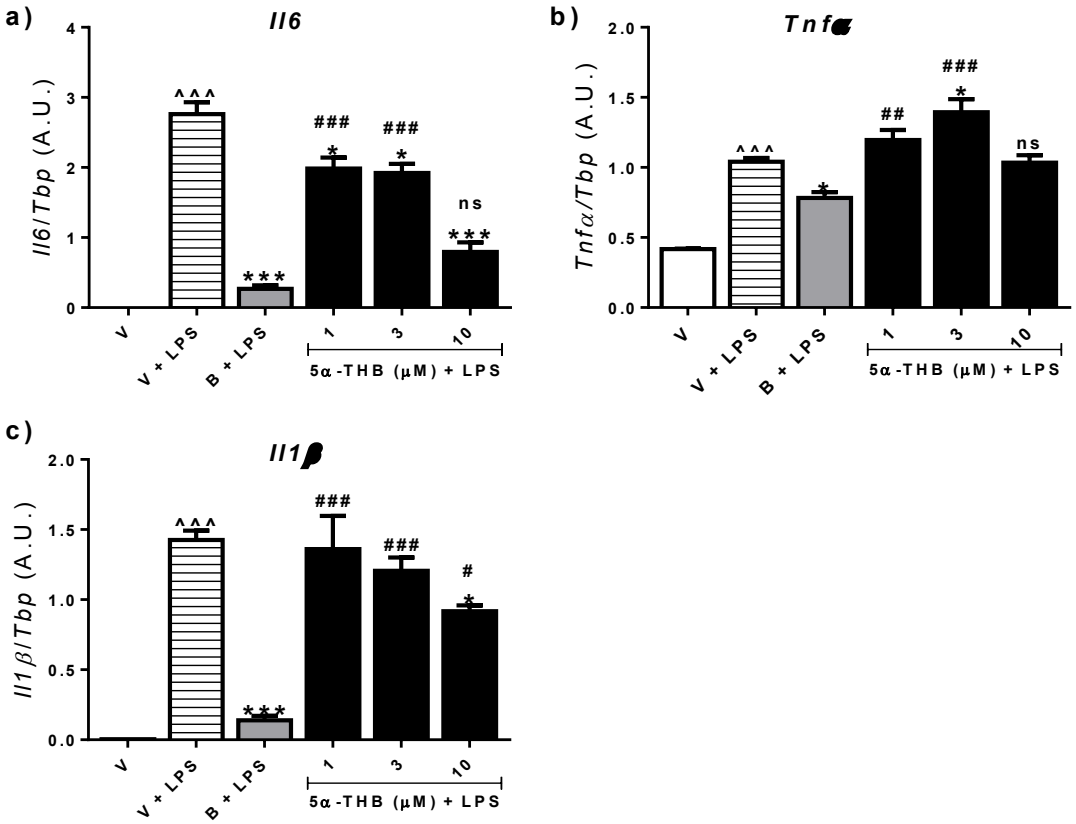

Figure 4.7 5α-Tetrahydrocorticosterone (5α-THB) decreased the abundance of transcripts of *Il6* and *Il1β* in cells stimulated with lipopolysaccharide (LPS). Quantification by real-time PCR of the abundance of transcripts of (a) Il6, (b) Tnfα and (c) Il1β following treatment with either vehicle (V), LPS (30 ng/mL) or corticosterone (B)/5α-THB + LPS for 24 hours. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ^^^ = $p < 0.0001$ vs V; *** = $p < 0.0001$, * = $p < 0.05$ vs V + LPS; ### = $p < 0.0001$, ## = $p < 0.001$, # = $p < 0.05$, ns = not significant vs B + LPS; N = 4. Tbp = TATA-box binding protein, A.U. = arbitrary unit.

4.3.3.3.3 Time-dependent production of pro-inflammatory cytokines in response to steroids

Some concentration-dependent effects of 5α-THB were seen employing a new batch of RAW264.7 cells. In order to analyse whether these results could be made more robust, the time-dependent production of IL6 and TNFα in response to treatment with LPS alone and in combination with steroids was investigated.

Production of IL6 increased in a time-dependent manner when cells were stimulated with LPS compared with the vehicle-treated group (Figure 4.8 a). Release of TNFα increased with the addition of LPS at every time point with the greatest increase seen at 4 and 8 h (Figure 4.8 b). Pre-treatment with B reduced the LPS-stimulated production of cytokines at 8, 16 and 24 h for IL6, and TNFα. An effect of 5α-THB was not seen at any time point tested.

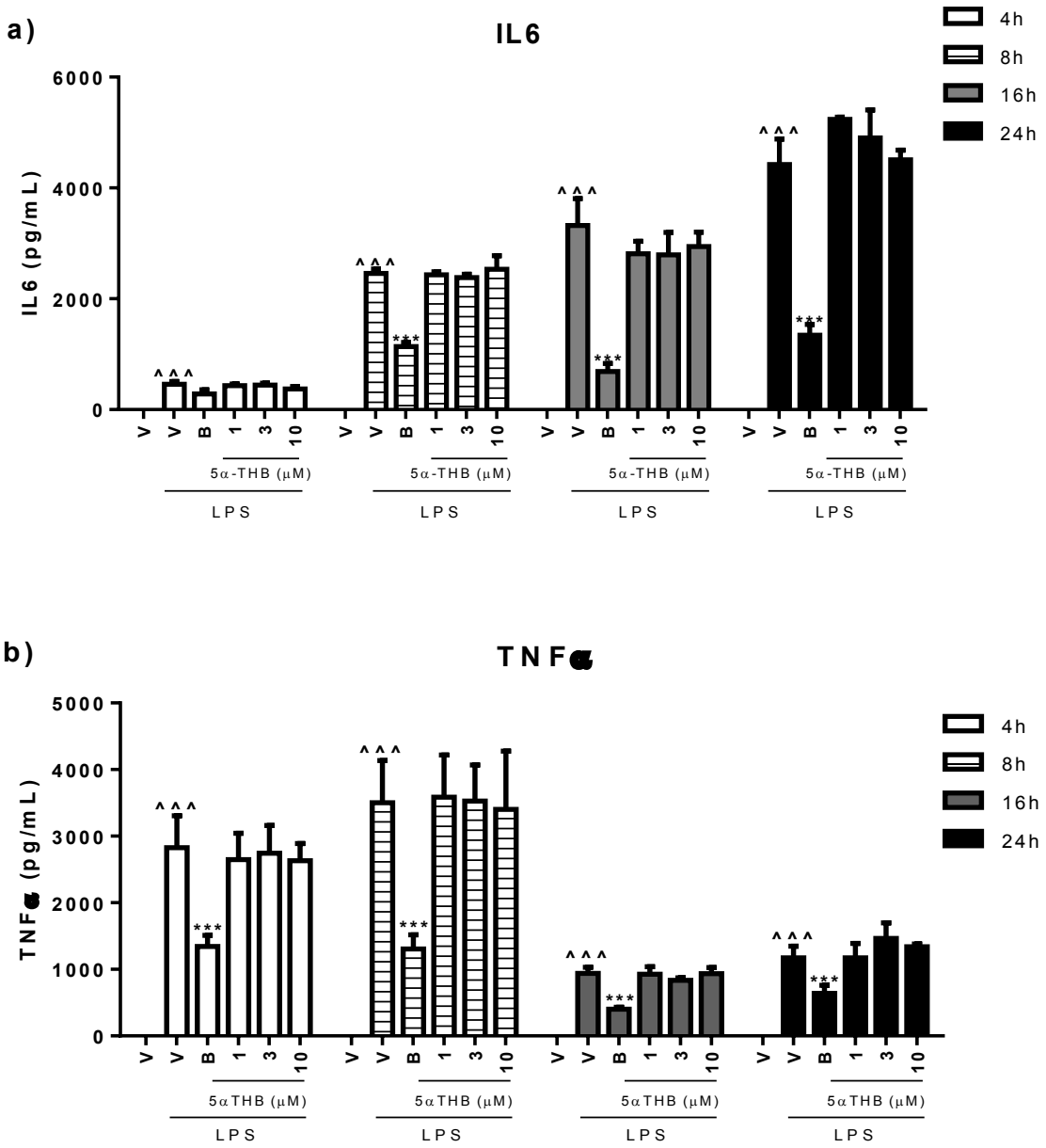

Figure 4.8 5α-Tetrahydrocorticosterone (5α-THB), in contrast to corticosterone (B), did not reduce the lipopolysaccharide (LPS)-stimulated production of cytokines. Time-dependent variations in the concentration of (a) IL6 and (b) TNFα as quantified by ELISA after treatment with either vehicle (V), LPS (30 ng/mL) alone or together with B (1 μM) or 5α-THB (1-3 μM). Data (mean ± SEM) were analysed with repeated measured ANOVA with Tukey's post-hoc test; ^^^ = $p < 0.001$ vs matched V; *** = $p < 0.0001$ vs matched LPS; N = 4.

4.3.4 Development of an *in vitro* model of inflammation using murine bone marrow-derived macrophages

The use of RAW264.7 cells proved challenging because of the variability of the results regarding the ability of 5α-THB to suppress the LPS-stimulated inflammatory response, with some evidence of the system/cells becoming less responsive with time. As a consequence, primary cells, murine bone marrow-derived macrophages (BMDMs) were employed as a complementary model.

Firstly, the production of IL6 and TNFα in response to increasing concentrations of LPS was investigated (Figure 4.9). Stimulation with LPS increased the concentration of both proteins compared with vehicle-treated cells when administered at a concentration equal to or greater than 1 ng/mL. For both cytokines, the greatest effect was achieved with a concentration of LPS of 3 ng/mL.

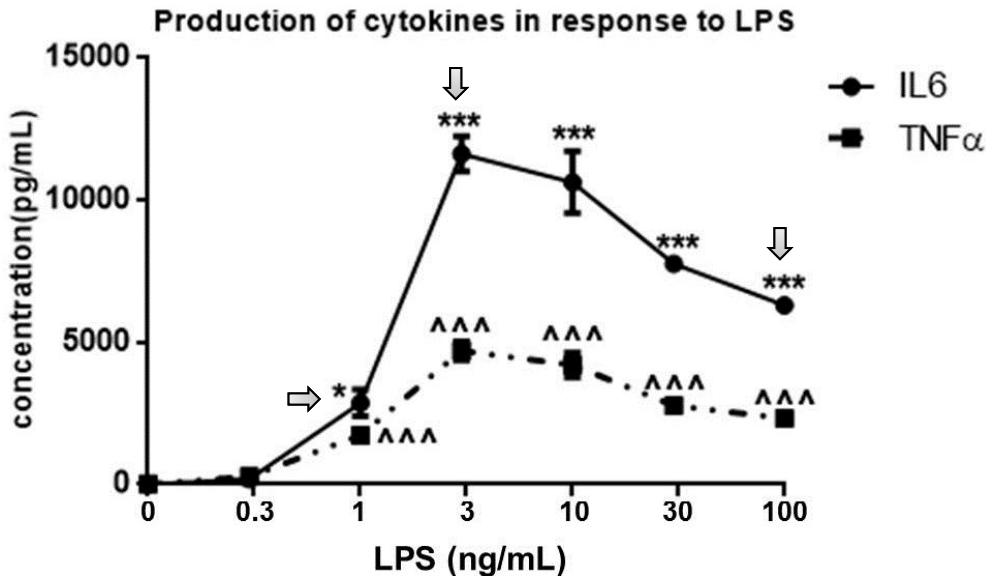

Figure 4.9 Concentration of cytokines by murine bone marrow-derived macrophages in response to stimulation with increasing concentrations of lipopolysaccharide (LPS). Quantification by ELISA of the concentration of IL6 and TNFα following stimulation with either vehicle (0 on the graph) or a range of concentrations of LPS. Data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; *** and ^^^ = $p < 0.0001$, * = $p < 0.05$ vs vehicle; N = 6. Arrows indicate the concentrations of LPS chosen for subsequent experiments. The graph was drawn as a semi-log plot; the log values were substituted with the real concentrations of LPS to give a clearer representation.

4.3.5 Ability of 5α-THB to suppress the inflammatory response

The response to 5α-THB, in comparison with that of B, was investigated using three different concentrations of LPS (1, 3 and 100 ng/mL), corresponding to three different responses in terms of the abundance of cytokines produced.

As shown in Figure 4.10, stimulation with every concentration of LPS increased the concentration of IL6 and TNFα compared with vehicle-treated cells. The largest increase was achieved with a concentration of 3 ng/mL for IL6 and of 3 and 100 ng/mL for TNFα. Treatment with B prior to LPS stimulation decreased the abundance of both cytokines compared with groups treated with any concentration of LPS. Treatment with 5α-THB decreased the concentration of IL6 compared with cells stimulated with LPS at 3 ng/mL, and reduced the abundance of TNFα increased by stimulation with LPS at 1 and 3 ng/mL. In both cases the greatest effect was achieved at a concentration of 5α-THB of 10 μM.

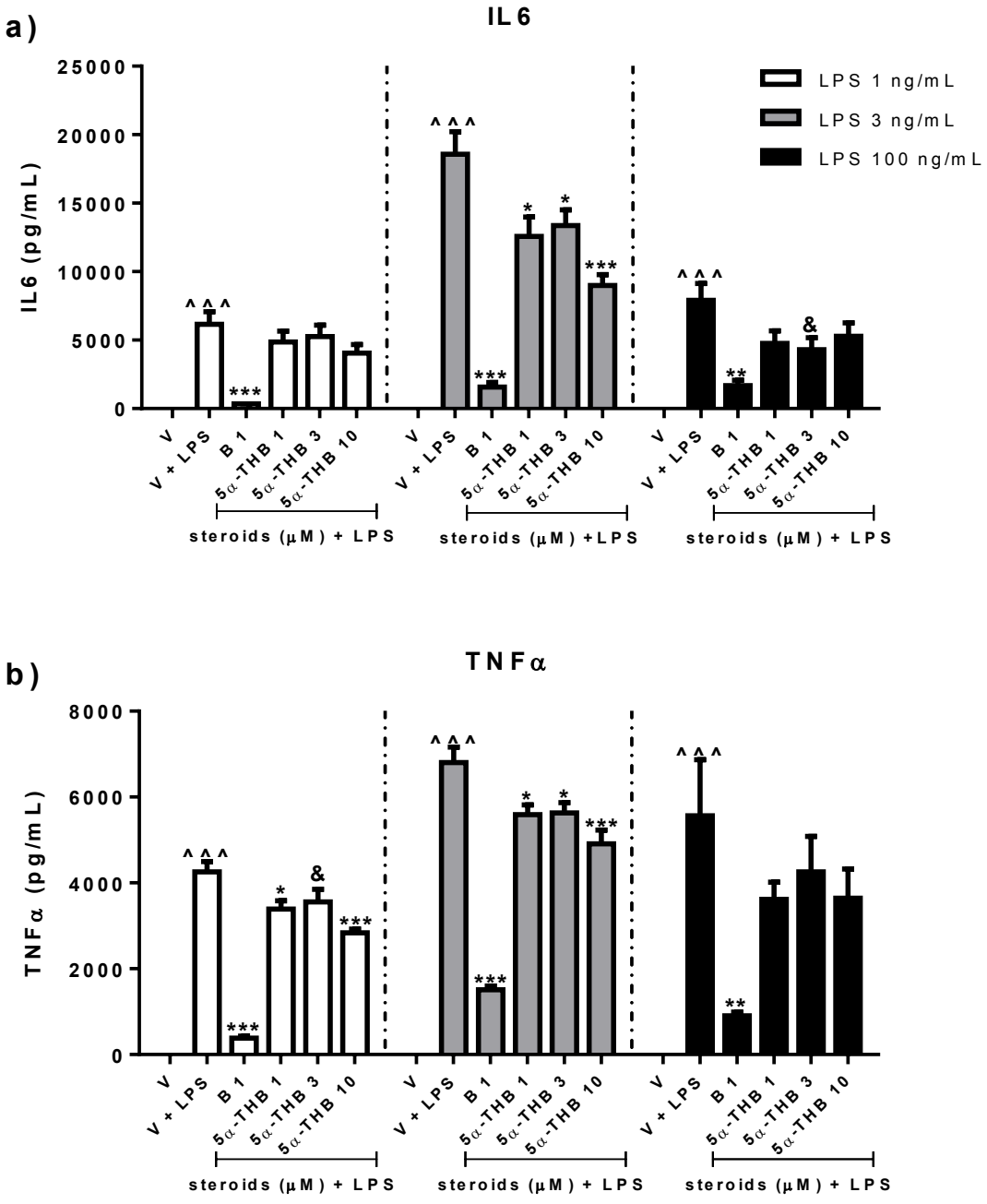

Figure 4.10 5α-Tetrahydrocorticosterone (5α-THB) reduced the lipopolysaccharide (LPS)-stimulated production of IL6 and TNFα in murine bone marrow-derived macrophages. Quantification by ELISA of the concentration of (a) IL6 and (b) TNFα following treatment with either vehicle (V) or a range of concentrations of LPS, with or without pre-incubation with either corticosterone (B) or 5α-THB. Data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; ^^^ = $p < 0.0001$ vs matched V; *** = $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$, & = $0.1 < p < 0.05$ vs matched V + LPS; N = 8.

4.3.6 Mechanisms of action of 5α-THB

In order to investigate the mechanisms whereby 5α-THB was reducing the production of cytokines in BMDMs, western blotting analysis of the signaling molecules IκBα and NF-κB was performed.

Treatment with LPS alone (1, 3 and 100 ng/mL) increased the abundance of both proteins compared with vehicle-treated cells (Figure 4.11 b, c). B decreased the abundance of NF-κB protein in cells stimulated with any concentration of LPS (Figure 4.11 b), while it increased the abundance of IκBα when cells were stimulated with 1 ng/mL of LPS, and decreased it when LPS was given at 100 ng/mL; the steroid had no effect in the group treated with LPS at 3 ng/mL (Figure 4.11 c). 5α-THB did not affect the abundance of NF-κB at any concentration of LPS (Figure 4.11 b), and decreased the abundance of IκBα only when cells were stimulated with LPS at 100 ng/mL (Figure 4.11 c).

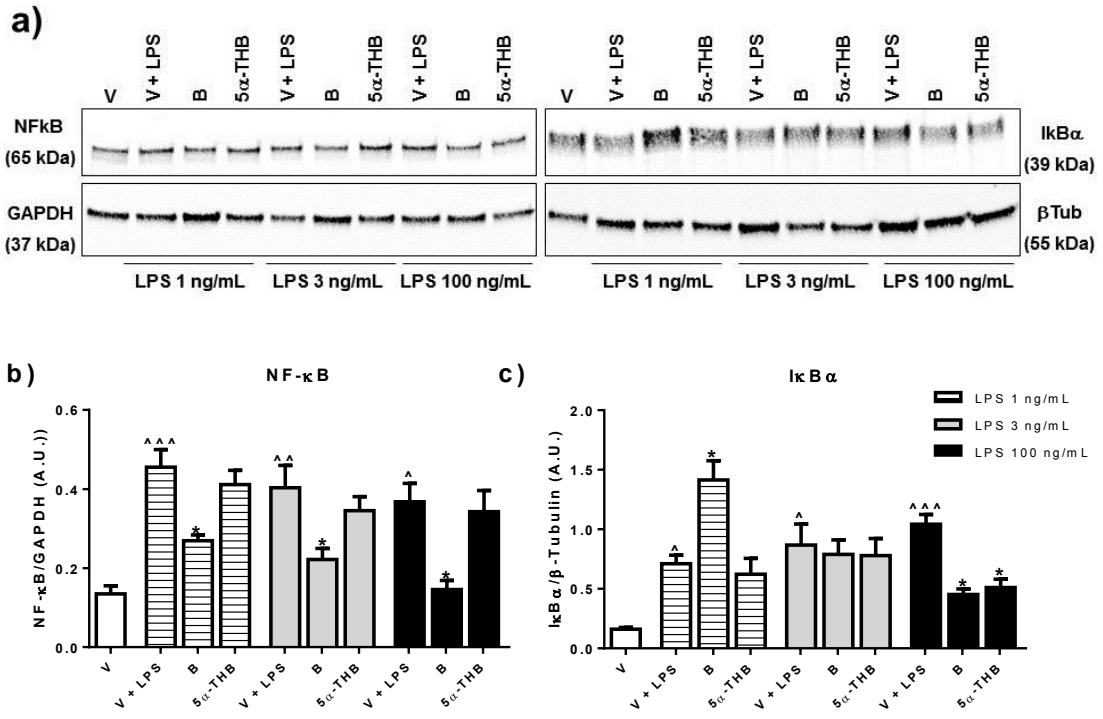

Figure 4.11 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) differentially affected the expression of IκBa and NF-κB in murine bone marrow-derived macrophages (BMDMs) stimulated with lipopolysaccharide (LPS). (a) Representative images of western blotting analysis for NF-κB (left-hand side) and IκBα (right-hand side) in BMDMs treated with vehicle (V) or stimulated with LPS (1, 3, 100 ng/mL) with or without pre-incubation with B (1 μM) or 5α-THB (10 μM); GAPDH = glyceraldehyde 3-phosphate dehydrogenase, βTub = βTubulin, kDa = kilo Dalton. (b) and (c) Quantification of western blotting analysis for the abundance of NF-κB and IκBα. Data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; * = $p < 0.05$ vs corresponding V + LPS; ^^^ = $p < 0.0001$, ^^ = $p < 0.001$, ^ = $p < 0.05$ vs V; N = 6; A.U. = arbitrary units.

4.3.7 Are the effects of 5α-THB mediated by GR?

In order to investigate whether the inhibitory effects of the steroids on the LPS-stimulated production of cytokines in BMDMs were mediated by GR, the abundance of IL6 in response to B and 5α-THB, in cells stimulated with LPS, were examined after pre-incubation with the GR antagonist RU486.

As shown in Figure 4.12, treatment with LPS (3 ng/mL) alone increased the concentration of IL6 in the medium compared with the vehicle-treated group; pre-incubation with RU486, followed by stimulation with LPS, decreased the abundance of the cytokine in a concentration-dependent manner compared with the LPS only-treated cells. When B (1 μM) was added alone before treatment with LPS, the amount of IL6 decreased compared with the group treated with LPS only; this decrease was partially reversed by the addition of RU486 at 1 μM. Treatment with 5α-THB alone (10 μM), before LPS stimulation, reduced the concentration of IL6 compared with the LPS only-treated group; the addition of increasing concentration of RU486 (0.01, 0.1, 1 μM) had an additive effect on that of 5α-THB. Incubation with RU486 alone at concentrations of 0.01, 0.1 and 1 μM reduced IL6 concentration to a similar extent to 5α-THB, 10 μM.

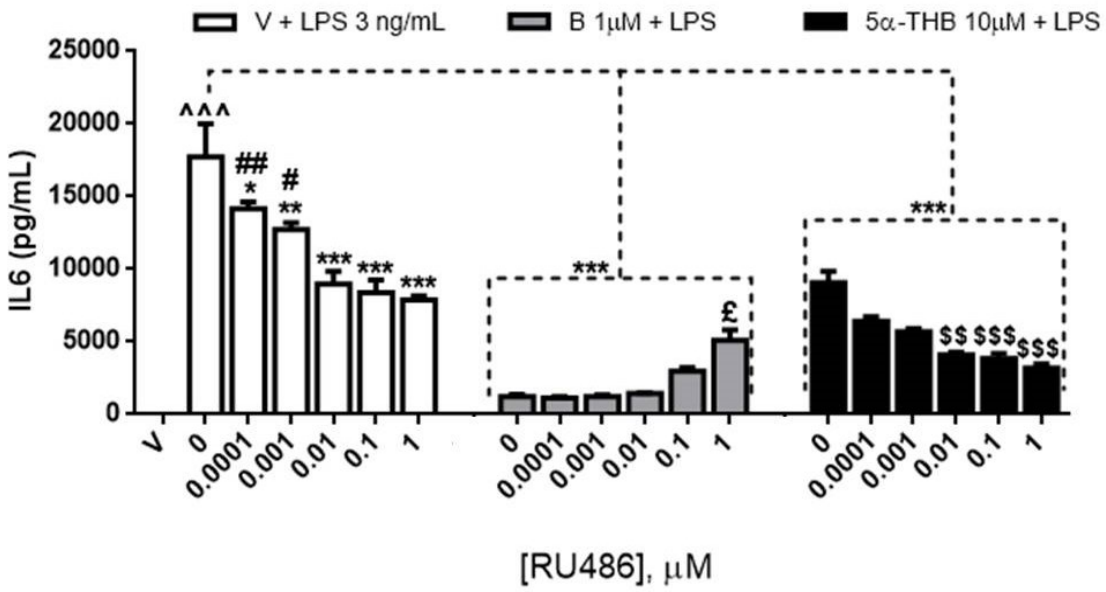

Figure 4.12 RU486 decreased the amount of IL6 released by lipopolysaccharide (LPS)-stimulated murine bone marrow-derived macrophages and this effect added to that of 5α-tetrahydrocorticosterone (5α-THB). Quantification by ELISA of the concentration of IL6 released after treatment with vehicle (V) or incubation with either LPS alone (3 ng/mL), or RU486 (concentrations as indicated) + LPS, or RU486 + B (1 μM) + LPS or RU486 + 5α-THB (10 μM) + LPS. Data (mean ± SEM) were analysed by one-way ANOVA followed by Tukey's post-hoc test; the dotted lines refer to the comparisons between cells treated only with LPS and those treated with LPS + steroids + increasing concentrations of RU486; ^^^ = $p < 0.0001$ vs V; *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ vs LPS alone (first white bar); £ = $p < 0.05$ vs B + LPS (first grey bar); $$$ = $p < 0.0001$, $$ and ## = $p < 0.001$, # $p < 0.05$ vs 5α-THB + LPS (first black bar); N = 6.

4.4 Discussion

In Chapter 3, 5α-THB was shown to have anti-inflammatory properties in an *in vivo* model of dermatitis in mice; surprisingly, experiments performed with the GR antagonist RU486 suggested that this receptor may not be the mediator of 5α-THB's effect. In order to explore these findings, simpler *in vitro* systems, widely in use in the literature for testing the anti-inflammatory potential of compounds, were employed in the present chapter. The utilisation of the murine macrophage cell line RAW264.7 proved challenging because of the lack of reproducibility in the results obtained. The employment of an alternative cell model, the culture of macrophages derived from bone marrow isolated from mice (BMDMs), gave consistent results, and showed that 5α-THB decreased the LPS-stimulated production of IL6 and TNFα. Furthermore, the investigation of possible mechanisms involved highlighted some differences between the ways whereby 5α-THB and B work. In effect, while the latter affected expression of IκBα and NF-κB and its action seemed dependent on GR, the former affected only the abundance of IκBα, and its behaviour was not antagonised by the GR antagonist RU486. This offers support for the lack of effect of this antagonist to attenuate the *in vivo* effects of 5α-THB shown in Chapter 3.

Macrophages are central players during the inflammatory response, and are capable of releasing a wide range of pro-inflammatory molecules which help orchestrate the response of the host to a wide range of insults, such as injury, infections and irritants (Novak and Koh 2013). GCs affect a broad spectrum of physiological processes, and are best known for their potent anti-inflammatory effects, brought about by their ability to interact either directly or indirectly with different cell populations of the immune system, among which are macrophages. *In vitro* models, while far from being perfectly representative of the complex inflammatory response occurring *in vivo*, are nonetheless useful tools for the investigation of the molecular pathways associated with inflammation. They have been used extensively (both in academia and industry) for dissection of the mechanisms through which GCs work and for the discovery and analysis of new promising anti-inflammatory agents. In addition, they allow for the study of pharmacological interventions in a simplified, controlled

environment, with the possibility of performing modifications of different parameters; this would be difficult in an *in vivo* setting. The murine RAW264.7 cell line has been widely employed as a steroid-responsive *in vitro* model for the study of a variety of putative or established anti-inflammatory compounds (Koo, Lee et al. 2004; Koo, Song et al. 2004; Rehman, Yoshihisa et al. 2012; Kenny, McCarthy et al. 2013; Shin, Ryu et al. 2013). In addition, previous studies in my supervisors' laboratory on the properties of 5α-THB were performed in this cell line, and to date, *in vivo* data have been obtained only from murine models. B was used as a positive control as its inhibitory effects on the production of cytokines are well-established in *in vivo* and *in vitro* models, including RAW264.7 cells (Xin, Zhang et al. 2011; Song, Zhao et al. 2013).

Initially, 5α-THB was shown to reduce the abundance of transcripts of genes encoding pro-inflammatory cytokines in RAW264.7 cells, albeit in a weaker fashion than B. However, when the same effects were investigated with higher concentrations of the compound, the results were not reproduced. As a consequence, the protocol used was questioned by testing a variety of experimental conditions; while in some of the experiments some effects of the compound were seen, the results did not point to one particular factor being responsible for the lack of reproducibility. On a technical note, the experiments looking at the time-dependent variations of abundance of transcripts for cytokines revealed different kinetics according to the gene analysed. These differences indicate that the analysis of different cytokines at the same time point may be not the ideal protocol to apply in the search for effects of a compound. An unexpected finding of the time-course studies was that after 16 hours of stimulation with LPS alone, the abundance of transcripts of inflammatory genes was not increased by this inflammatory stimulus. This result proved challenging to explain, also after a review of the literature. It has been reported that the release of cytokines from alveolar macrophages, and the inhibitory effects of dexamethasone, were dependent on culture methodology (Higham, Lea et al. 2014), and even though this research pointed to a more pronounced difference in unstimulated cells, the finding suggests that macrophages may be particularly susceptible to culture conditions. In addition, at the same point in

time (16 h), B and 5α-THB seemed to have pro-inflammatory effects. A dual role of GCs in controlling inflammation has been previously shown (Zhong, Wang et al. 2013; Cruz-Topete and Cidlowski 2014). In particular, in peritoneal macrophages activated with LPS, dexamethasone regulated transcription of pro-inflammatory cytokines in an opposite manner depending on its concentration, increasing it at low concentration and decreasing it at high concentration (Lim, Muller et al. 2007). Despite some of the results with the RAW264.7 cells pointing to a pro-inflammatory behaviour of 5α-THB, the data were not reproducible, and therefore their interpretation is not possible.

The employment of a new batch of RAW264.7 cells initially provided promising results as 5α-THB reduced in a concentration-dependent manner the abundance of transcripts of *Il6*, but this did not translate into an inhibition of the release of the cytokine in the medium. The demonstration, in subsequent experiments employing BMDMs, that 5α-THB consistently suppressed the production of IL6 and TNFα, suggests that the lack of reproducibility in RAW264.7 cells may lie in the overall lack of suitability of the model system itself.

The use of primary BMDMs for the investigation of macrophage biology is an excellent tool; cells are freshly isolated and differentiated for each experiment. While this may result in a degree of variability due to the intrinsic differences between animals, it also has the advantage of providing the researcher with a fresh source of macrophages (Weischenfeldt and Porse 2008). Since BMDMs have been employed in my supervisors' laboratory in previous studies analysing 5α-THB, a protocol was already available (Yang 2009). However, it was decided to investigate the response of these cells to different concentrations of LPS in order to determine the best experimental conditions, particularly in light of the results obtained with the RAW264.7 cell model. The production of TNFα was generally lower than that of IL6, and they reached different maximal responses. This mirrored results obtained previously with RAW264.7 cells after incubation with 30 ng/mL of LPS for 24 hours. It was decided to study the effects of 5α-THB using three concentrations of LPS, representing three different aspects of the BMDMs concentration-response

curve obtained: 1 ng/mL which induced a low-moderate response, 3 ng/mL which stimulated the maximal response and 100 ng/mL which corresponded to a production of IL6 in between the two concentrations; in the literature the suggested concentration is in the 10-100 ng/mL range, but it is recognised that the response of macrophages varies according to the protocol employed, the source of LPS and its purity. Consequently, the optimal range must be determined empirically (Mosser and Zhang 2008). The ability of 5α-THB to suppress the release of IL6 and TNFα varied according to the cytokine and the concentration of LPS investigated; in contrast, B lowered the production of both cytokines under any experimental condition tested, indicating the existence of differences regarding the mechanisms whereby the two compounds work to reduce pro-inflammatory responses. In Chapter 3, no effects of 5α-THB were seen on the abundance of transcripts for IL6 and TNFα in inflamed ears. It is possible that the compound may affect the production of the cytokines also in the dermatitis model by acting at post-transcriptional level, but this mechanism has not been studied yet; however, it also likely that differences between the *in vivo* situation and the *in vitro* model may account for divergent effects of 5α-THB.

To clarify which mechanisms may be differentially influenced by the two steroids, analysis of the expression of the transcription factor NF-κB and the signalling molecule IκBα was performed. Stimulation with LPS only increased the abundance of NF-κB at any concentration, with a pattern resembling that of the production of TNFα. It may well be that TNFα was responsible for inducing part of the augmented expression of NF-κB (De Bosscher, Vanden Berghe et al. 2003; Vandevyver, Dejager et al. 2012). The amount of IκBα was also increased by all concentrations of LPS. As mentioned in the introduction to this chapter, inflammatory stimuli activate downstream molecules such as NF-κB and AP-1 to up-regulate the expression of pro-inflammatory mediators. Between the time when the cell encounters a stimulus and the activation of such transcription factors, other intermediate events take place. They depend on the activity of molecules such as p38 MAPK and IκBα; the latter binds to NF-κB in the cytoplasm and impedes its translocation to the nucleus when the cell is in a resting state (De Bosscher, Vanden Berghe et al. 2003). Upon

inflammatory stimulation, a protein kinase phosphorylates IκBα inducing its degradation, and so freeing NF-κB. The results presented here indicate that incubation with LPS may promote the release of inflammatory cytokines by increasing also the abundance of NF-κB protein, in addition to promoting its translocation to the nucleus. The increase of IκBα with LPS may seem contradictory given the pro-inflammatory role of LPS and the anti-inflammatory action of IκBα. However, it may represent a negative feed-back to avoid an excessive inflammatory response. Indeed, activation of NF-κB by TNFα is followed by the recruitment of co-activators or co-repressors to the IκBα gene, in a proportion that is cell- and situation-dependent (Gao, Chiao et al. 2005).

The presence of B decreased NF-κB in all experimental conditions, and increased the abundance of IκBα when BMDMs were stimulated with LPS at 1 ng/mL, and decreased it when LPS was given at 100 ng/mL. In contrast, 5α-THB did not affect the amount of NF-κB, and only decreased IκBα expression in cells stimulated with 100ng/mL of LPS. It is thought that GCs mainly affect NF-κB by inhibiting its binding to DNA regulatory sequences by a tethering mechanism, and *de novo* protein synthesis seems not to play a big role in their ability to suppress the production of pro-inflammatory cytokines (De Bosscher, Schmitz et al. 1997; Wissink, van de Stolpe et al. 1997). However, this chapter showed that B also affected NF-κB at the transcriptional level. While no effects of 5α-THB on the abundance of NF-κB protein were revealed, there is still the possibility that it may inhibit the ability of NF-κB to bind to DNA. Studies investigating the cellular distribution of NF-κB in response to steroids may help to reveal more about the mechanism of action of 5α-THB. The increase of IκBα seen with B at the lowest LPS concentration is in accordance with its anti-inflammatory properties, and it is an effect that has been already documented (Clark 2007; Newton and Holden 2007), while the decrease at the highest LPS concentration was unexpected. The phenomenon was seen also with 5α-THB. As puzzling as these results may appear, since they point to pro-inflammatory behaviour, it needs to be remembered that the mechanisms analysed in this chapter represent only a fraction of the processes activated by LPS, and steroids

work by affecting many of them. Moreover, the possibility that GCs may have pro-inflammatory properties is not unprecedented. In addition, investigating whether the 5α-reduced steroid affect the abundance of the phosphorylated form of IκBα, which is sensitive to degradation, may shed light on whether the compound modifies the extent to which NF-κB is freed through alteration of the degradation of the sequestering protein. Other signalling molecules such as MAPKs, involved in the AP-1 pathway, could also be examined.

The search for the possible molecular mechanisms underlying the effects of 5α-THB, and explaining the differences with B, was directed next to the investigation of whether the compound acts via GR. Surprisingly, the GR antagonist alone decreased the production of IL6 from BMDMs stimulated with LPS – no precedent for this could be found in the literature but a clear concentration-response was evident. Despite being used mainly as an antagonist of the GR, in *in vivo* and *in vitro* models, RU486 is known to have also agonist properties, delivered by both its targets, the progesterone receptor (PR) and GR (Beck, Estes et al. 1993; Beck, Weigel et al. 1993; Zhang, Jonklaas et al. 2007; Chien, Lai et al. 2009); in effect, the compound is classified as a type II antagonist because it induces a conformation of the receptor which resembles that promoted by the binding of the agonist, and so has mixed agonist/antagonist characteristics (Moguilewsky and Philibert 1984; Gravanis, Schaison et al. 1985; Horwitz 1985; Antonakis, Markogiannakis et al. 1991; Beck, Estes et al. 1993; Sartorius, Groshong et al. 1994; McDonnell, Dana et al. 1995; Schulz, Eggert et al. 2002). The agonist properties of RU486 have been studied mainly regarding trans-activation of genes (Schulz, Eggert et al. 2002; Zhang, Jonklaas et al. 2007), but the compound has also been shown to repress some non-genomic and genomic cellular responses; for instance, when given alone to human T cells, it blocked proliferation induced by phytohemagglutinin (PHA), and when administered with progesterone, it enhanced its anti-proliferative properties (Chien, Lai et al. 2009); in addition, RU486 was shown to inhibit at a pre-translational level, and in a concentration-dependent manner, the expression of the interleukin-2 receptors in HPA-stimulated human lymphocytes, therefore mimicking the effect of dexamethasone (Antonakis, Markogiannakis et al. 1991). RU486 bound to GR has

also been found capable of inducing transcription of genes synergistically with the signalling molecule cAMP, again acting in a similar way to dexamethasone in immature murine T cells (Beck, Weigel et al. 1993). An interesting study showed that the extent of the agonistic activity of RU486 on induction of the MMTV-Luc reporter was determined not by the efficiency with which GR bound to the compound interacted with target DNA sequences, but rather by the abundance of the co-repressor NCoR (nuclear receptor co-repressor) in the cellular environment (Schulz, Eggert et al. 2002). This indicates that RU486 may behave differently in different cell types, according to the availability of co-repressors. Thus, it can be speculated that in BMDMs the amount of NCoR was such that an agonistic function of RU486 was encouraged.

The results obtained with co-incubation of B and RU486 suggest an antagonist effect of the compound. This is what is classically expected, and is consistent with B working through GR. An intriguing result was that 5α-THB and RU486 together had cumulative inhibitory effects on the abundance of IL6. This phenomenon was seen before in similar experiments performed in my supervisors' laboratory using a single concentration of RU486 (1 μM) (Yang 2009). This may be explainable in different ways. First of all, the two steroids may be binding to different populations of the same receptor, perhaps GR, and, as a consequence, be having additive effects. In support of this possibility is the fact that RU486 binds GRβ (Lewis-Tuffin, Jewell et al. 2007). The fact that 5α-THB is a weaker suppressor of cytokine production than B, and possibly RU486, may have encouraged this scenario. However, another possibility is that the compounds may be targeting different receptors. Nuclear receptors such as PR and LXR are present in macrophages, and display anti-inflammatory features dependent on repression of the activity of transcription factors such AP-1 (Hong and Tontonoz 2008; Dressing, Goldberg et al. 2010; Huang and Glass 2010; Perissi, Jepsen et al. 2010; Schulman 2010); furthermore, RU486 can have PR-dependent agonist properties (Zhang, Jonklaas et al. 2007; Chien, Lai et al. 2009). These results highlight the importance to include controls in experiments in order to correctly interpret the outcomes. They also suggest that a pure antagonist may be more suitable for clarifying whether GR is involved in the response triggered

by steroids; unfortunately, at the moment such a compound is not available. However, another strategy would be to knock down GR expression by siRNA, and this is an approach that is planned by the supervisors' group.

In summary, in this chapter 5α-THB was shown to suppress pro-inflammatory cytokine production in BMDMs in a weaker manner than B, and through mechanisms that do not seem to entail a strong regulation of the expression of NF-κB and IκBα, in contrast to B. More importantly, the 5α-reduced steroid may not work through a conventional interaction with GR, as already suggested by *in vivo* experiments in Chapter 3.

Chapter 5

EFFECTS OF 5α-THB ON ANGIOGENESIS

Chapter 5: Effects of 5α-THB on angiogenesis

5.1 Introduction

The effects of GCs on the vasculature are multifaceted. On the one hand, their anti-inflammatory properties can be explained in part by the fact that they inhibit the expression of molecules responsible for the activation and hyper-permeability of the vasculature seen during inflammation (i.e. VEGFα, TNFα, IL6, ICAM1). On the other hand, their negative effects on the proliferation of endothelial cells and production of extracellular matrix components such as collagen, cause, in the long term, inhibition of angiogenesis and wound healing in the skin (Christian, Graham et al. 2006; Hengge, Ruzicka et al. 2006; Sanchis, Alba et al. 2012; Tiganescu, Tahrani et al. 2013). These aspects make GCs desirable but risky drugs to use, most of all when they are applied for a long period of time. As a consequence, the ideal compound for the treatment of skin conditions such as eczema or psoriasis would be one that, while inhibiting inflammation by affecting also the vasculature (e.g. by inhibiting expression of adhesion molecules), would spare other processes such as angiogenesis. In Chapter 3, the anti-inflammatory properties of 5α-THB, and the mechanisms underpinning them, were investigated in a model of skin inflammation. The compound was found to decrease the abundance of transcripts of genes involved in vascular changes associated with inflammation, and in particular with recruitment of inflammatory cells at the site of injury. While this represents a promising therapeutic profile, more research is needed in order to unveil the effects of 5α-THB on the formation of new vessels, and therefore bring to light the potential for vascular side effects.

In this chapter, the effects of 5α-THB on angiogenesis were studied using a murine *in vivo* model in which the subcutaneous implantation of a sponge induces the development of a granulomatous response including intense formation of new vessels and the infiltration of inflammatory cells (Ferreira, Barcelos et al. 2004). This model, therefore, allowed the contemporaneous investigation of how two systems closely related, the vasculature and the immune system, respond to administration of compounds.

Previous studies (Small, Hadoke et al. 2005; Logie, Ali et al. 2010) suggested that the inhibitory effect of GCs on angiogenesis is dependent on GR. Having demonstrated in previous chapters that the anti-inflammatory actions of 5α-THB were not prevented by the use of a GR antagonist, and taking into account previous published and unpublished work which highlighted the lack of side effects caused by 5α-THB on skin thickness and metabolic parameters (Yang, Nixon et al. 2011), the hypothesis for this chapter is that:

- 5α-THB does not inhibit angiogenesis in subcutaneously implanted sponges in mice but is able to reduce the inflammatory response.

Objectives

To determine whether 5α-THB inhibits: a) the formation of new vessels and b) the inflammatory response in sub-cutaneous sponge implants in mice.

5.2 Materials and Methods

5.2.1 Murine *in vivo* model of angiogenesis

5.2.1.1 Preparation of sponges and silastic pellets

5.2.1.1.1 Materials

Polyurethane sponge grade XE1700V was used for all experiments and they were a kind gift from Caligen Foam Ltd. (Accrington, Lancashire, U.K.). For delivery of steroids, silastic pellets were made using SILASTIC® MDX4-4210, BioMedical Grade Elastomer, obtained from Dow Corning, MIDLAND, USA. Steroids were purchased from Steraloids (Newport, RI, USA).

5.2.1.1.2 Preparation of sponges

Cubic sponges (approximately 0.5 cm^3) were cut manually from a bigger pad. They were autoclaved and stored at RT prior to subcutaneous implantation on flanks of mice.

5.2.1.1.3 Preparation of implants of inert silicone elastomer (silastic pellets)

Silastic pellets have been shown to release steroids at a constant rate for up to 4 weeks (Cleasby, Livingstone et al. 2003). For the preparation of silastic pellets to use as vehicle (control pellets), curing agent was mixed with base elastomer in a ratio of 1:10 by weight, and subjected to centrifugation (10 s, 16000 x *g*). For pellets containing corticosterone (B) or 5α-THB, 250 mg of base elastomer were mixed with 100 mg of steroid and subjected to centrifugation (10 s, 16000 x *g*) prior to addition of 25 mg of curing agent followed by another round of centrifugation (10 s, 16000 x *g*). The mixture was left to cure (RT, 48 h) before removal from the container; the pellet was divided in smaller pellets so that each contained the amount of steroids indicated in the "Result" section of this chapter. Pellets were inserted into the sponge using fine forceps under sterile conditions in a laminar flow cabinet prior to subcutaneous implantation.

5.2.1.2 Subcutaneous implantation of sponges

5.2.1.2.1 Materials

All surgical tools were from Fine Science Tools (Linton, UK).

5.2.1.2.2 Surgery

Surgical instruments were autoclaved prior to surgery and maintained sterile during the procedure by using a Novasapa cold steriliser. Male C57BL/6 mice were weighed prior to administration of anaesthesia (isofluorane-vet; Merial, Harlow, UK) by inhalation; once animals were deemed unconscious, local analgesic (Vetergesic multidose, Alstoe, York, UK; 0.05 mg/kg) was given by subcutaneous injection. Animal were shaved on the neck region, and a longitudinal incision of about 0.5 cm was made using fine scissors. In order to create two subcutaneous pouches where the sponges could be implanted, a pair of curved, blunt forceps was passed through the neck incision and a tunnel created under the skin on each side of the mouse. Sponges were inserted using fine forceps so that two sponges were implanted in each mouse, one in each flank. The incision was closed with surgical clips and mice allowed to recover from anaesthesia in a clean cage. After recovery, mice were kept in groups of three for 21 days, after which they were euthanized by decapitation always between 8 and 10 a.m. to decrease the variability between outcome measures of experiments due to the circadian variations in endogenous corticosterone level. Biological materials and implants were recovered as described in section 5.2.1.5.

5.2.1.2.3 Experimental groups

The control (or vehicle) group consisted of mice implanted with two sponges, one in each flank, containing a pellet without steroids (vehicle pellets). Steroid-treated groups consisted of mice implanted with sponges containing pellets loaded with steroids; these were always implanted on the right flank of the mouse, while the left received sponges containing vehicle pellets as internal control for systemic effects of the steroids.

5.2.1.3 Blood collection and plasma preparation

Blood was collected at two time points: a) two days prior to euthanasia tail blood was collected with the help of Dr. Dawn Livingstone or Tracy Mak in lithium heparin-treated tubes (Fisher Scientific, Northumberland, UK) in order to analyse the basal concentration of corticosterone, and b) at cull trunk blood was collected in tubes treated with sterile EDTA solution (0.5M, pH 8). Plasma was recovered as described in section 2.2.3.

5.2.1.4 Quantification of B in plasma

The concentration of B in plasma was measured as described in section 2.3.3.4.

5.2.1.5 Recovery of sponges and biological materials

Sponges were recovered from their flanks and divided into two halves: one half was immersed in RNA later for RNA extraction and real-time PCR (sections 2.3.1.3, 2.3.1.5, 2.3.1.6) while the other was placed into formalin (10% v/v in PBS; Sigma-Aldrich, Dorset, UK) and processed for either H&E staining as described in section 2.3.4, or for immunostaining as described in section 4.2.2.1.

Thymus and adrenal glands were collected and placed in 10% formalin (RT, 24 h) prior to transfer in 70% ethanol; 48 h later their weights were measured using a precision scale.

5.2.2 Analysis of sponges and tissues

5.2.2.1 Histological analysis

5.2.2.1.1 Materials

All materials were provided by the Histology facilities of the Shared University Research Facilities (SuRF), Little France Campus, Edinburgh and were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Primary antibodies were: rabbit polyclonal anti-CD31 (Abcam, Cambridge, UK), rat monoclonal to F4/80 (eBiosciences, Hatfield, UK) and mouse monoclonal anti-αSMA (Sigma-Aldrich, Dorset. UK). Secondary antibodies were included in the Leica Refine Detection Kit (Leica biosystems, Milton Keynes, UK) supplied for the Leica Staining Robot.

Picrosirius solution: Fast Green (0.1%, 50 mL), Direct Red (0.1%, 50 mL), Picric Acid Solution (900 mL).

5.2.2.1.2 H&E staining

H&E staining of sections of sponge was performed as described in section 2.3.4.

5.2.2.1.3 Picrosirius (PSR) staining for collagen fibres

PSR staining was performed by Ms Debbie Mauchline of the Histology Facilities of the Shared University Research Facilities (SuRF), Little France Campus, Edinburgh. Paraffin was removed and samples were hydrated as described for H&E staining

(section 2.3.4). After a wash in running tap water, they were immersed in PSR solution (2 h), de-hydrated and mounted as described (section 2.3.4.3).

5.2.2.1.4 Immunostaining for CD31, αSMA and F4/80

Immunostaining against the antigens CD31, αSMA and F4/80 was performed by Ms Ruth Hamblin of the Histology facilities of the Shared University Research Facilities (SuRF), Little France Campus, Edinburgh. Immunostaining was carried out using a Leica Staining Robot, with antigen retrieval for F4/80 performed robotically using trypsin solution (0.5 mg/mL in PBS, (Sigma-Aldrich), 10 min, 37 °C), or manually using a pressure cooker for αSMA under standard conditions (citrate NCL pH6 buffer, Sigma-Aldrich); no antigen retrieval was performed for CD31. For F4/80 and αSMA, samples were blocked (5 min) with hydrogen peroxide solution from the Leica Refine Detection Kit (Leica biosystems, UK) followed by serum block (30 min; F4/80, Immpress anti rat (mouse absorbed) kit (Vector Laboratories, Peterborough, UK); αSMA, Mouse on Mouse Abcam Kit (Abcam, Cambridge, UK)). Thereafter, samples were incubated with primary antibody (F4/80 1:300; αSMA 1:4000; 30min), prior to incubation with polymer (30 min, F4/80 Impress Kit as above; αSMA Abcam Kit as above). Immunostaining was completed with incubation with 3,3'-diaminobenzidine (DAB, 10 min) and counterstaining with haematoxylin (5 min), both from the Leica Refine Kit. For CD31 immunostaining a Leica Refine Kit (as above) was used, which included a hydrogen peroxide block (5 min), primary antibody (120 min, 1:200) and polymer incubation (15 min) followed by DAB (10 min) and haematoxylin (5 min).

5.2.2.1.5 Quantification of the number of vessels in sponges

In order to quantify angiogenesis, the number of vessels present in the implanted sponges was counted. The person counting was blinded to treatment. Vessels in sections stained with H&E were recognized by their round appearance and the presence of erythrocytes in the lumen. The number of vessels positive for CD31 or αSMA staining was also investigated by counting every tubular structure showing reactivity to DAB (brown stain). Three sections from each sponge were examined under a microscope (Axioskop, Zeiss, Germany) and vessels counted in the field of

vision at magnification of 100X. Analysis of the data was performed taking into consideration the mean value of the three cross sections for each sponge.

5.2.2.1.6 Quantification of cells infiltrating the sponges

Total cells infiltrating the sponges were quantified by counting under the microscope (magnification 200X) the number of blue nuclei present in three different fields of vision in sections stained with H&E. Quantification of macrophages was carried out with the same method but by using a higher magnification (400X) and by counting the number of cells positive for the marker F4/80 and the total number of cells (blue nuclei and cells positive for F4/80 staining) in the field of vision.

5.2.2.1.7 Quantification of collagen staining

The intensity of Picrosirius staining on pictures taken at a magnification of 100X was quantified using the free imaging program ImageJ (NIH, USA). A Macro was kindly developed and provided by Mr. James Baily.

5.2.2.2 Molecular analysis

5.2.2.2.1 RNA isolation

Total RNA was isolated from sponges as described in section 2.3.1.3.

5.2.2.2.2 Real-time PCR analysis

Production of cDNA and subsequent real-time PCR analysis were performed as described in sections 2.3.1.5 and 2.3.1.6.

5.2.3 Data analysis

5.2.3.1 General

Data were represented as mean ± SEM and analysed as described in section 2.4.

5.2.3.2 Weights of the adrenal glands and thymus

The weights of the adrenal glands and thymus were represented as absolute weights measured at cull.

5.2.3.3 Real-time PCR analysis

For quantification of transcripts through real-time PCR, the abundance of each gene was represented in relation to the abundance of chosen housekeeping genes. The two

housekeeping genes used were *Tbp* and *Gapdh* due to the lack of significant changes in the abundance of transcripts following treatment with steroids. For each sample and each gene studied the values presented in the graphs were calculated as follow: value sample X/mean values *Tbp* + *Gapdh* sample X.

5.3 Results

5.3.1 Development of an *in vivo* model of angiogenesis

5.3.1.1 Qualitative evaluation of angiogenesis in sponges

Implanted sponges retrieved after 20 days *in situ* showed clear signs of angiogenesis during visual examination (Figure 5.1 A). They appeared to have an intense red colour consistent with the growth of vessels and presence of red blood cells. Furthermore, they were encapsulated by connective tissue. Staining with H&E (Figure 5.1 B) confirmed the presence of vessels (black arrows in the picture) surrounded by a fibrous stroma containing cell nuclei (stained pink and purple, respectively). Other features visible were pink triangular shapes which represented pieces of the sponge (blue arrow in picture).

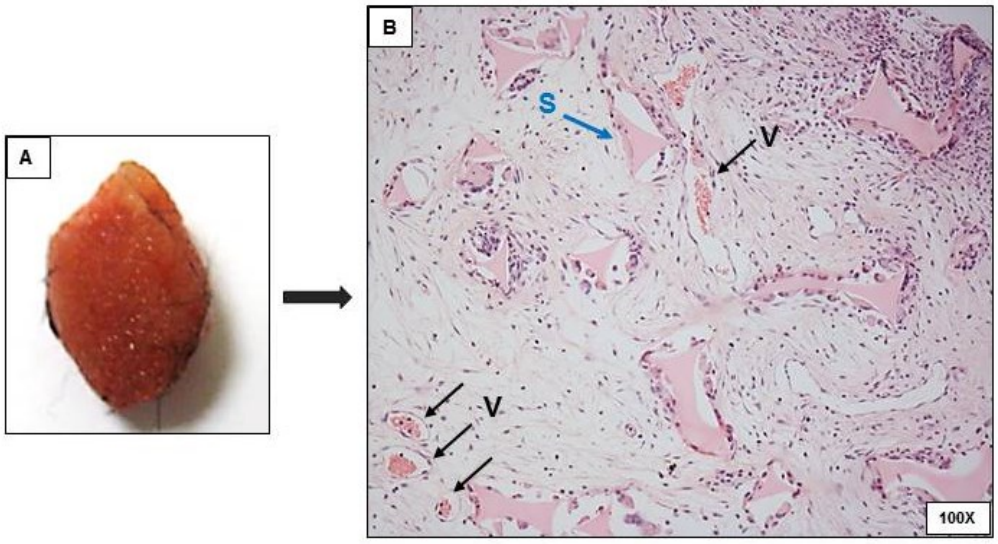

Figure 5.1 Angiogenesis took place in sponges implanted subcutaneously in mice for 20 days. (A) Representative macroscopic image of a sponge recovered after 20 days *in situ* from the flank of a mouse. (B) Representative image of a cross-section of a sponge stained with H&E. Black arrows point to vessels of different calibre containing visible red blood cells; V = vessels. The blue arrow points to a piece of material that constitutes the sponge (S = sponge). The vessels and the sponge material are surrounded by tissue and cell nuclei (stained pink and purple, respectively). Magnification = 100X.

5.3.2 Effects of steroids on angiogenesis using the sponge model

5.3.2.1 Systemic effects of local administration of steroids

In order to investigate whether the local administration of steroids via pellets inserted into the implants had systemic effects, the concentration of B in blood and the weights of adrenal glands and thymus were analysed.

The amount of circulating B was not different between the experimental groups (Figure 5.2 a), both at 19 days and at cull. The presence of B in sponges decreased the weight of the adrenal glands but not of the thymus compared with the control group (Figure 5.3 a and b). 5α-THB did not exert an effect on either of them.

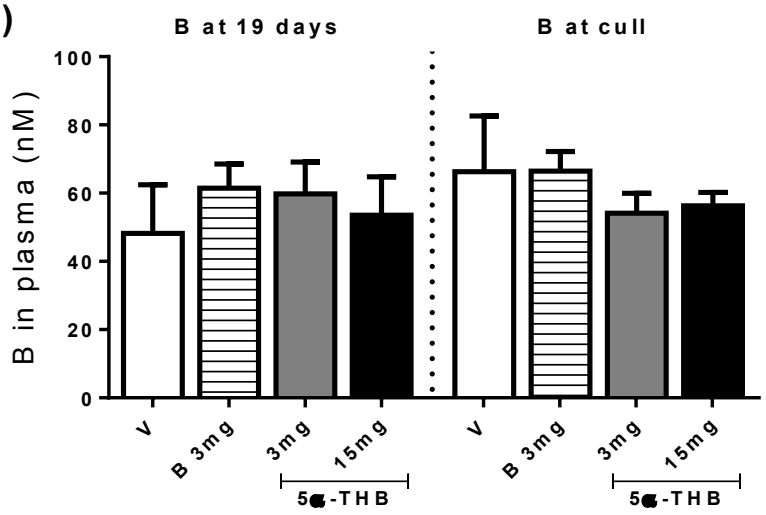

Figure 5.2 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) in sponges did not affect the concentration of circulating B. Concentration of B (nM) quantified in blood taken either at 19 days after implantation of sponges (left-hand side) or at cull after 20 days (right-hand side). Mice received the treatments indicated; V = control sponges. Data are mean ± SEM analysed with One-way ANOVA; n = 6-9 mice/group.

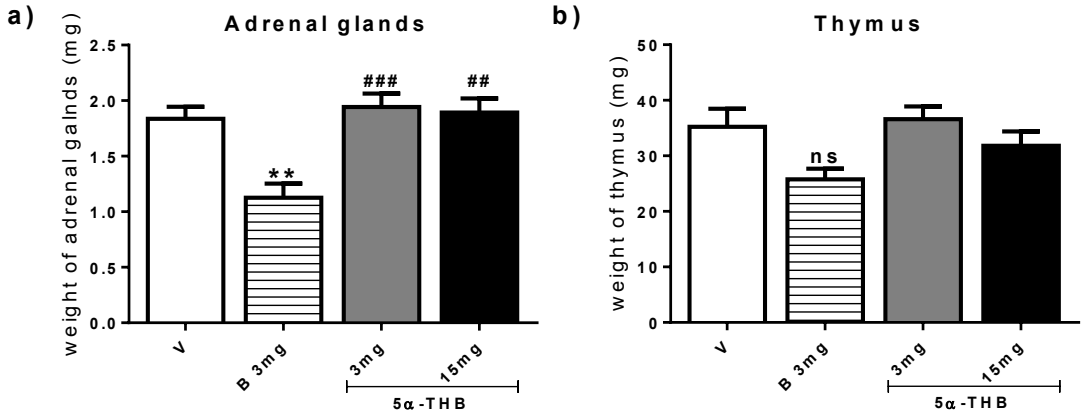

Figure 5.3 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not affect the weight of the adrenal glands. Absolute weights of (a) adrenal glands and (b) thymus in mice implanted with sponges containing the indicated treatments. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. ** = $p < 0.001$, ns = not significant vs V; ### = $p < 0.0001$, ## = $p < 0.001$ vs B 3mg; n = 8-9 mice/group.

5.3.2.2 Ability of 5α-THB to inhibit local angiogenesis

5.3.2.2.1 Macroscopic analysis of sponges

Visual analysis of control sponges (containing vehicle pellets) collected from untreated mice revealed an appearance consistent with growth of vessels and presence of red blood cells (Figure 5.4 a, b); the gross appearance was very similar to that of sponges not containing any pellet (Figure 5.1 A). A connective tissue capsule surrounding the sponge was also present. Control sponges implanted in the left flanks of mice receiving steroid treatment on the right also appeared vascularised (Figure 5.4 c, e, g). However, control sponges from mice receiving B showed a less dense external fibrous capsule (Figure 5.4 c).

A clear difference was noted when sponges loaded with 3 mg B were examined (Figure 5.4 d). These sponges were much paler in colour, resembling pre-implantation sponges, and were lacking the connective capsule; these characteristics suggested reduced growth of vessels and tissue. Evaluation of sponges containing 3 mg of 5α-THB (Figure 5.4 f) showed that this treatment did not induce major visual changes compared with the control group. A slight discoloration of the sponges, and some lack of connective tissue around them, were noted when pellets were loaded with 15 mg of 5α-THB (Figure 5.4 h) suggesting a dose-dependent effect on the growth of vessels and tissue.

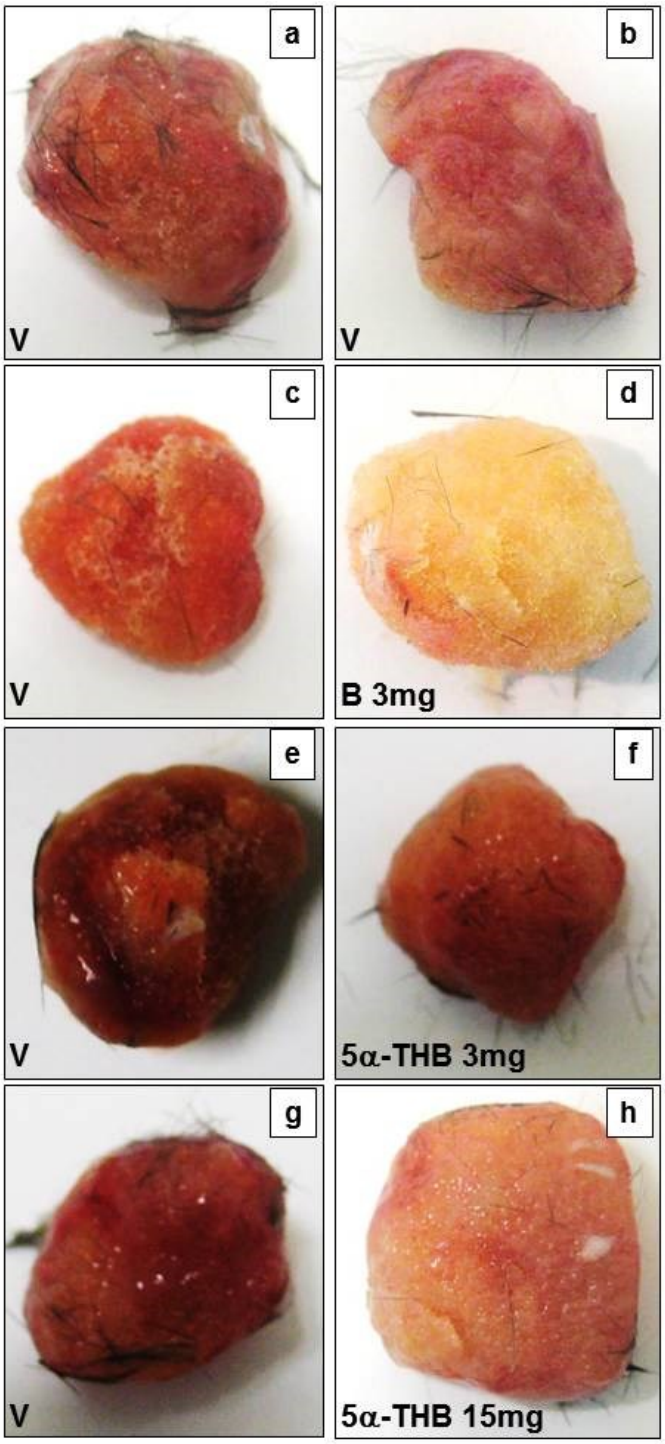

Figure 5.4 5α-Tetrahydrocorticosterone (5α-THB) caused visual changes of sponges consistent with inhibition of angiogenesis to a lesser extent than corticosterone (B). Representative pictures of sponges collected from the flanks of mice after 20 days *in situ*, and containing either vehicle pellets (V) or pellets loaded with either B (3 mg) or 5α-THB (3 mg, 15 mg) as indicated.

5.3.2.2.2 Microscopic analysis of sponges

Microscopic analysis of sections of sponges implanted in control mice, and stained with H&E, revealed the presence of a conspicuous number of tubular structures (red arrows in Figure 5.5 a, b) containing red blood cells (RBC in Figure 5.5 b), indicating that angiogenesis has taken place. These structures were visible mainly on the outer edges of the sponges and differed regarding the calibre (see Figure 5.5 b for a clearer vision at higher magnification). Purple cell nuclei could be discerned throughout the sections (blue arrows) and also inside the blood vessels. Structurally, the control sponges appeared to have a looser central part, mainly formed by the material constituting the sponge itself (S in Figure 5.5 a) and loose fibrous tissue, whilst all around this core, a more dense fibrous capsule was present.

Qualitative analysis of sponges loaded with B at 3 mg (Figure 5.5 d) showed a decreased number of vessels, which also appeared to have a reduced calibre, and a looser fibrous structure throughout the sponge. Cell nuclei were still visible, although they seemed less abundant compared with the control sponges (a, b). Staining of control sponges implanted on the left flank of these mice (Figure 5.5 c) revealed a number of vessels comparable with that of the control group. However, the central part of these sponges seemed to have a less dense fibrous structure.

Microscopic analysis of sponges loaded with 5α-THB at 3 mg (Figure 5.5 f) showed a structure and an abundance of vessels that appeared similar to the control sponges (a, b). Sponges containing pellets with 5α-THB at 15 mg (Figure 5.5 h) had smaller and less abundant vessels compared with the control group. In both cases, a higher number of cell nuclei compared with the group treated with B seemed to be present. The appearance of sponges implanted in the contra-lateral flank as controls in both of these groups (Figure 5.5 e, g) was comparable to the control group.

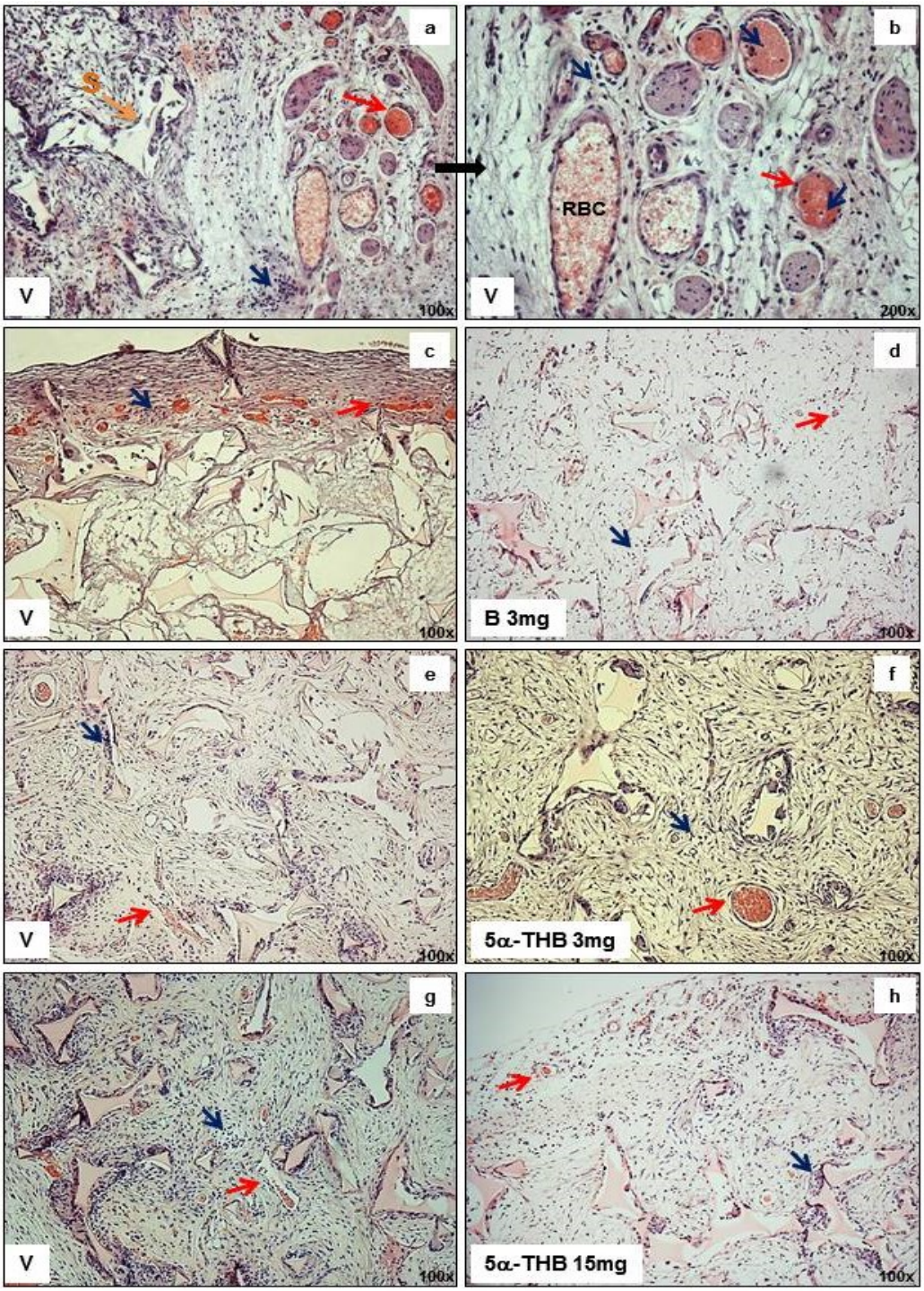

Figure 5.5 5α-Tetrahydrocorticosterone (5α-THB) affected the microscopic appearance of the sponges to a lesser extent than corticosterone (B). Representative pictures of sponges loaded with vehicle pellets (V) or with pellets containing either B (3 mg) or 5α-THB (3 mg, 15 mg) as indicated. Red arrows = vessels; blue arrows = cell nuclei; RBC = red blood cells; S = sponge matrix. Magnification = 100X except for (b) = 200X.

5.3.2.2.3 Quantitative analysis of angiogenesis in sponges

The number of vessels (Figure 5.6 a) was lower in sponges containing B (3 mg) and 5α-THB at 15 mg compared with the control group (V). In sponges loaded with 5α-THB at 3 mg the number of vessels showed a statistical trend towards significance; this was the case also for control sponges from mice receiving B and 5α-THB at 15mg.

In order to avoid confounding results, and since the main interest of this study was to investigate the local and direct effect of steroids, subsequent analyses were performed excluding the vehicle contra-later sponges. As a consequence, the effect of steroids was statistically re-analysed and represented as a percentage of the control group set to 100% (Figure 5.6 b); B was found to decrease angiogenesis by 85.0 ± 3.3% while both concentrations of 5α-THB (3 and 15 mg) decreased the abundance of vessels by approximately 25.0 ± 7.4% and 55 ± 7.6%, respectively.

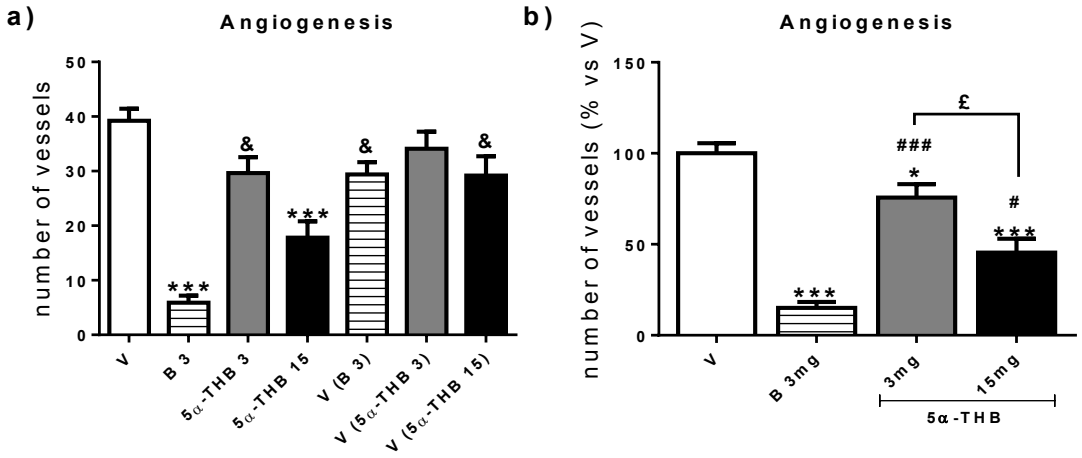

Figure 5.6 5α-Tetrahydrocrticosterone (5α-THB) reduced angiogenesis in sponges, but was less potent than corticosterone (B). (a) Number of vessels in sponges. The quantity (in mg) of steroids is reported as number after their names. The last three groups represent the contra-lateral control sponges implanted in mice receiving treatment with the indicated steroid. (b) Evaluation of angiogenesis as percentage of the control group (mice not receiving steroid) set to 100%. V = control group. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. *** = $p < 0.0001$, * = $p < 0.05$, & = $0.1 < p < 0.05$ vs V; ### = $p < 0.0001$, # = $p < 0.05$ vs B 3; £ = $p < 0.05$; n = 8-12 mice/group.

5.3.2.3 Effects of 5α-THB on different aspects of the angiogenic process

5.3.2.3.1 Analysis of vessels expressing the endothelial marker CD31

Immunostaining for the endothelial marker CD31 (Figure 5.7) showed the presence of vessels of different calibre (orange arrows in the Figure) in each of the experimental groups. The staining allowed the recognition of tiny vessels not clearly discernible with H&E staining. Furthermore, some of the vessels appeared to be undergoing a process of branching or duplication (arrows containing an asterisk in Figure 5.7) suggesting that angiogenesis was taking place. The visual examination indicated that the abundance and the calibre of the vessels positive for CD31 staining were reduced in the sponges containing B and 5α-THB at 15 mg (Figure 5.7 (b) and (d)) compared with the control group (V, (a)).

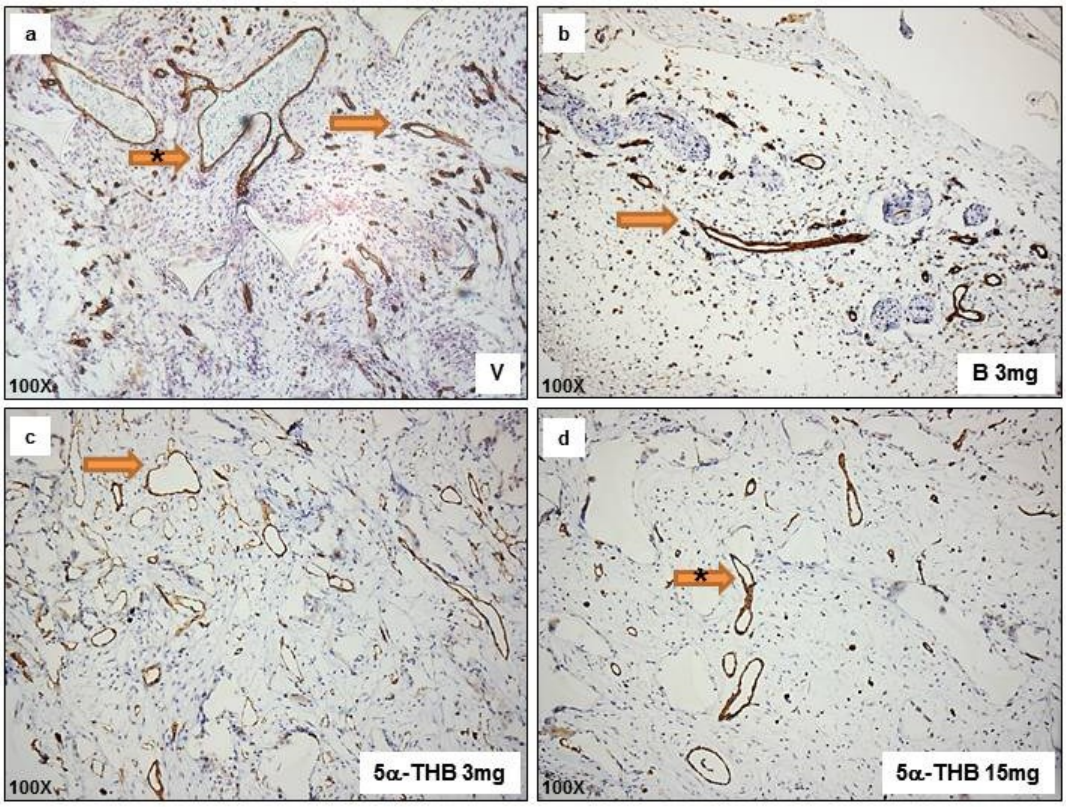

Figure 5.7 5α-Tetrahydrocorticosterone (5α-THB, 15 mg) and corticosterone (B) appeared to reduce the abundance of vessels expressing the endothelial marker CD31. Representative pictures of sections of sponges stained for the endothelial marker CD31. V = control group. Orange arrows point to vessels positive for CD31 staining; those with an asterisk indicate vessels undergoing a process of duplication or branching. Magnification = 100X.

Quantification of the number of vessels stained for CD31 showed that treatment with B (3 mg) and 5α-THB at 15 mg, but not at 3 mg, reduced the number of CD31-positive vessels compared with the control group (Figure 5.8 a). When the amount of CD31-positive vessels in each group was represented as a percentage compared with the control group set to 100% (Figure 5.8 b), B reduced this amount by 98 ± 0.7%, while 5α-THB at 15 mg brought it down by 80 ± 5.3%.

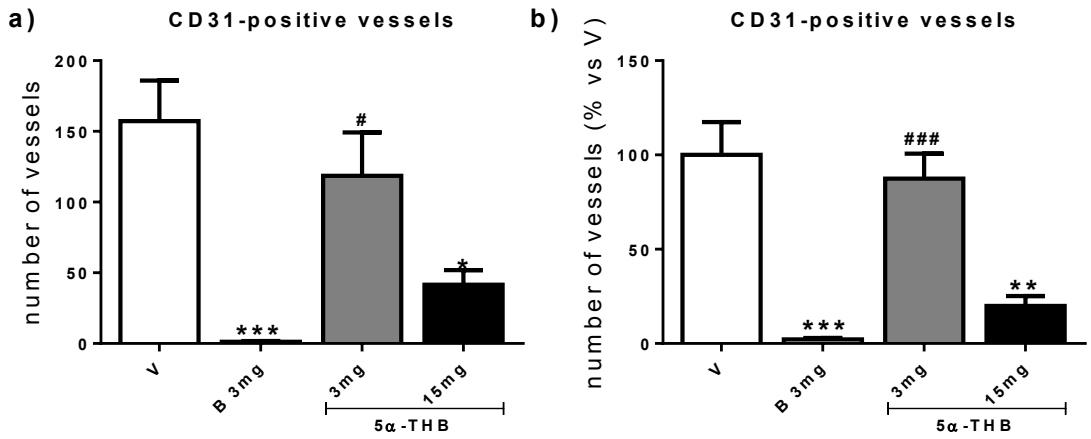

Figure 5.8 5α-Tetrahydrocorticosterone (5α-THB, 15 mg) and corticosterone (B) reduced the abundance of vessels positive for the endothelial marker CD31. (a) Quantification of the number of vessels positive for the endothelial marker CD31; (b) % of the number of vessels positive for CD-31 compared with the control group set to 100%. V = control group. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$; ### = $p < 0.0001$, # = $p < 0.05$ vs B 3mg; n = 8-12 mice/group.

5.3.2.3.2 Analysis of vessels expressing alpha smooth muscle actin (αSMA)

Immunostaining for the marker of smooth muscle cells αSMA revealed the presence of vessels of different calibre (blue arrows), as seen with previous staining. The intensity and distribution of the staining, and the abundance of vessels, looked similar between the control group (Figure 5.9 a) and the groups treated with 5α-THB (Figure 5.9 c, d). However, samples of the group treated with B showed reduced staining intensity compared with the control group (Figure 5.9 b), and fewer tube-like structures, indicating a decrease in the number of vessels expressing αSMA.

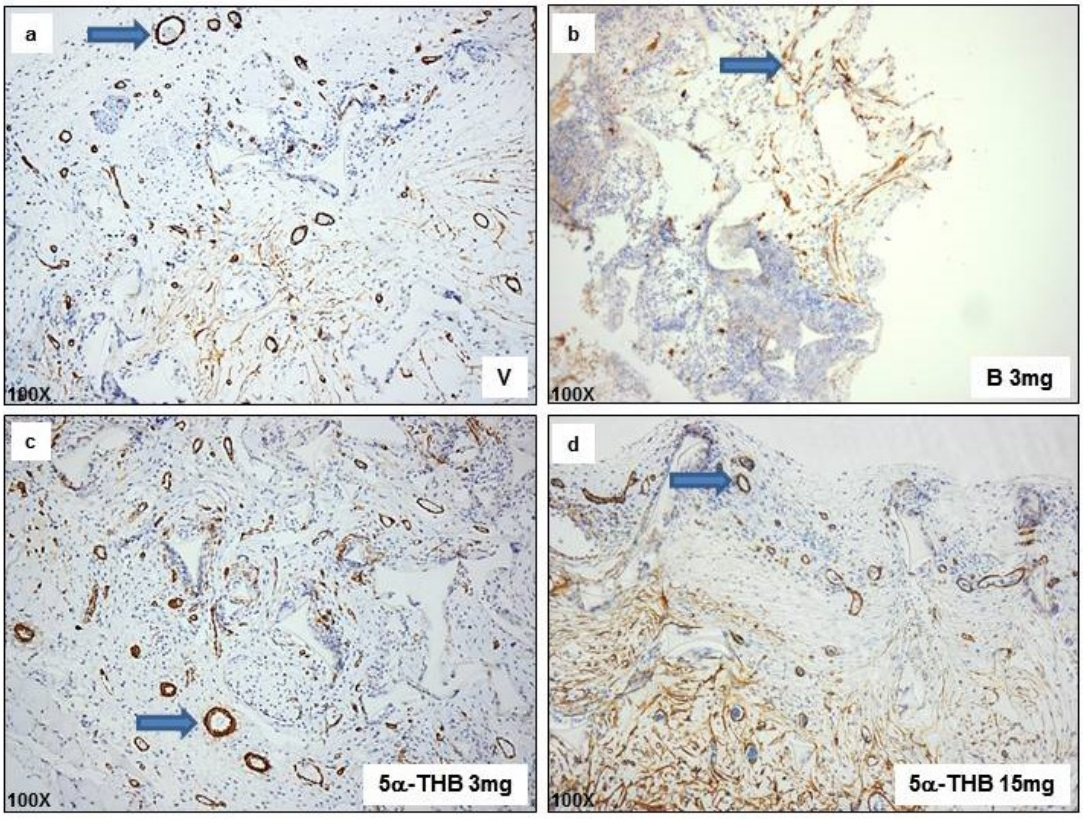

Figure 5.9 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not appear to reduce the number of vessels expressing the smooth muscle marker αSMA. Representative pictures of sections of sponges stained for the smooth muscle marker αSMA. V = control group. Blue arrows point to vessels positive for αSMA staining. Magnification = 100X.

Quantification of the number of vessels stained for αSMA showed that only treatments with B (3 mg) reduced the absolute number compared with the control group (Figure 5.10 a). Representation of this analysis as a percentage of αSMA-positive vessels (Figure 5.10 b) revealed that B decreased the abundance by approximately 88 ± 3% compared with the control group set to 100%. 5α-THB at 15 mg reduced the number of positive vessels by approximately 44 ± 14%; however, this reduction was not statistically significant compared with the control group.

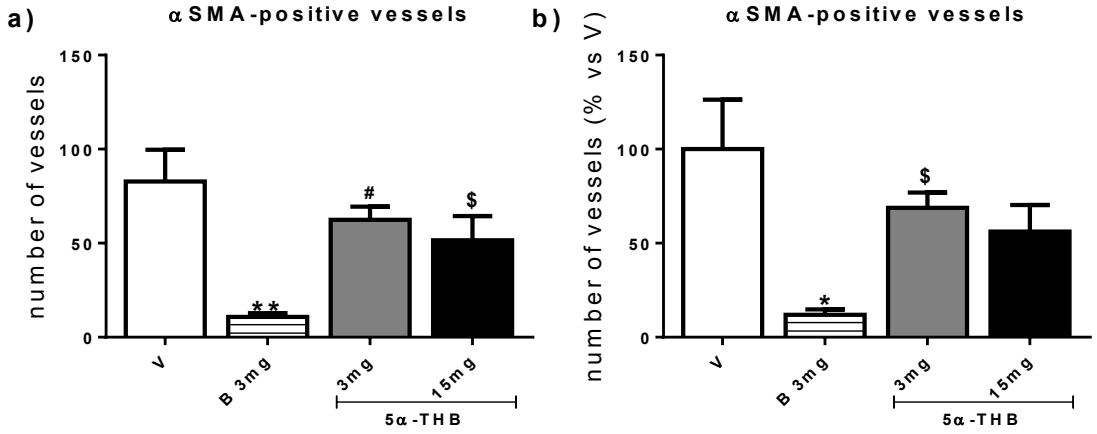

Figure 5.10 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not reduce the abundance of vessels positive for the smooth muscle marker αSMA. (a) Quantification of the number of vessels positive for the smooth muscle marker αSMA; (b) % of the number of vessels positive for αSMA compared with the control group set to 100%. V = control group. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. ** = $p < 0.001$, * = $p < 0.05$ vs V; # = $p < 0.05$, $ = $0.1 < p < 0.05$ vs B 3mg; n = 8-12 mice/group.

5.3.2.3.3 Analysis of cell infiltration in sponges

5.3.2.3.3.1 Total cell number

The count of the total number of cell nuclei in sponges stained with H&E (Figure 5.11 a) revealed that treatment with B and 5α-THB (15 mg) decreased the number of cells compared with the control group. The effect was similar between the two steroids, as B reduced the cell number by approximately 40 ± 7.3%, and 5α-THB by 45 ± 3.3 compared with the control group set to 100% (Figure 5.11 b).

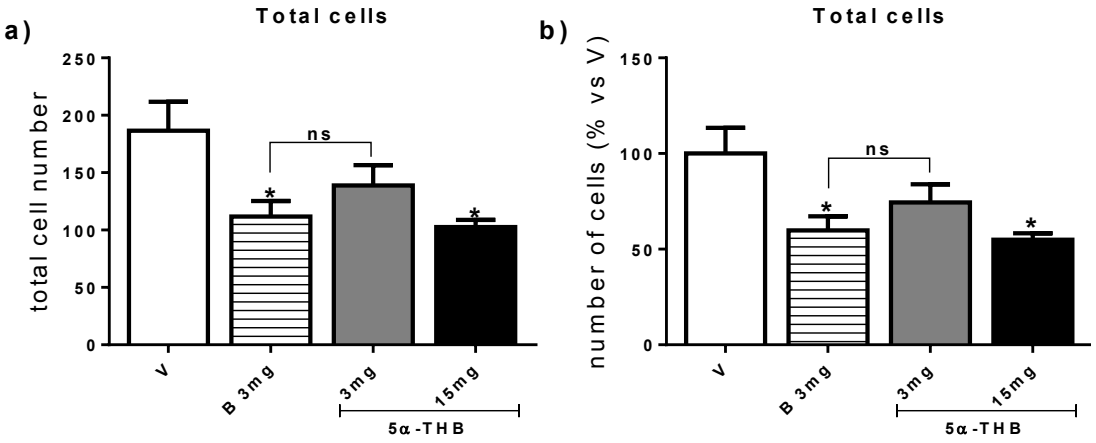

Figure 5.11 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) reduced the number of total cells infiltrating the sponges to a similar extent. (a) Quantification of the total number of cell nuclei in sections of sponges; (b) total number of cell nuclei represented as percentage of the control group set to 100%. V = control group. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. * = $p < 0.05$ vs V; n = 8-12 mice/group.

5.3.2.3.3.2 Cell expressing the macrophage marker F4/80

Immunostaining for cells expressing the protein F4/80 (Figure 5.12, purple arrows) revealed that sponges containing B (3mg) and 5α-THB (15 mg) (Figure 5.10 b, d) appeared to have a reduced amount of cells positive for the marker compared with the control group (Figure 5.12 a). Groups treated with 5α-THB at 3 mg did not show major differences at the visual examination (Figure 5.12 c). Quantification of the proportion of cells positive for F4/80 (Figure 5.13) confirmed that the groups treated with B and 5α-THB, 15 mg, decreased the proportion of macrophages to a similar extent compared with the control group.

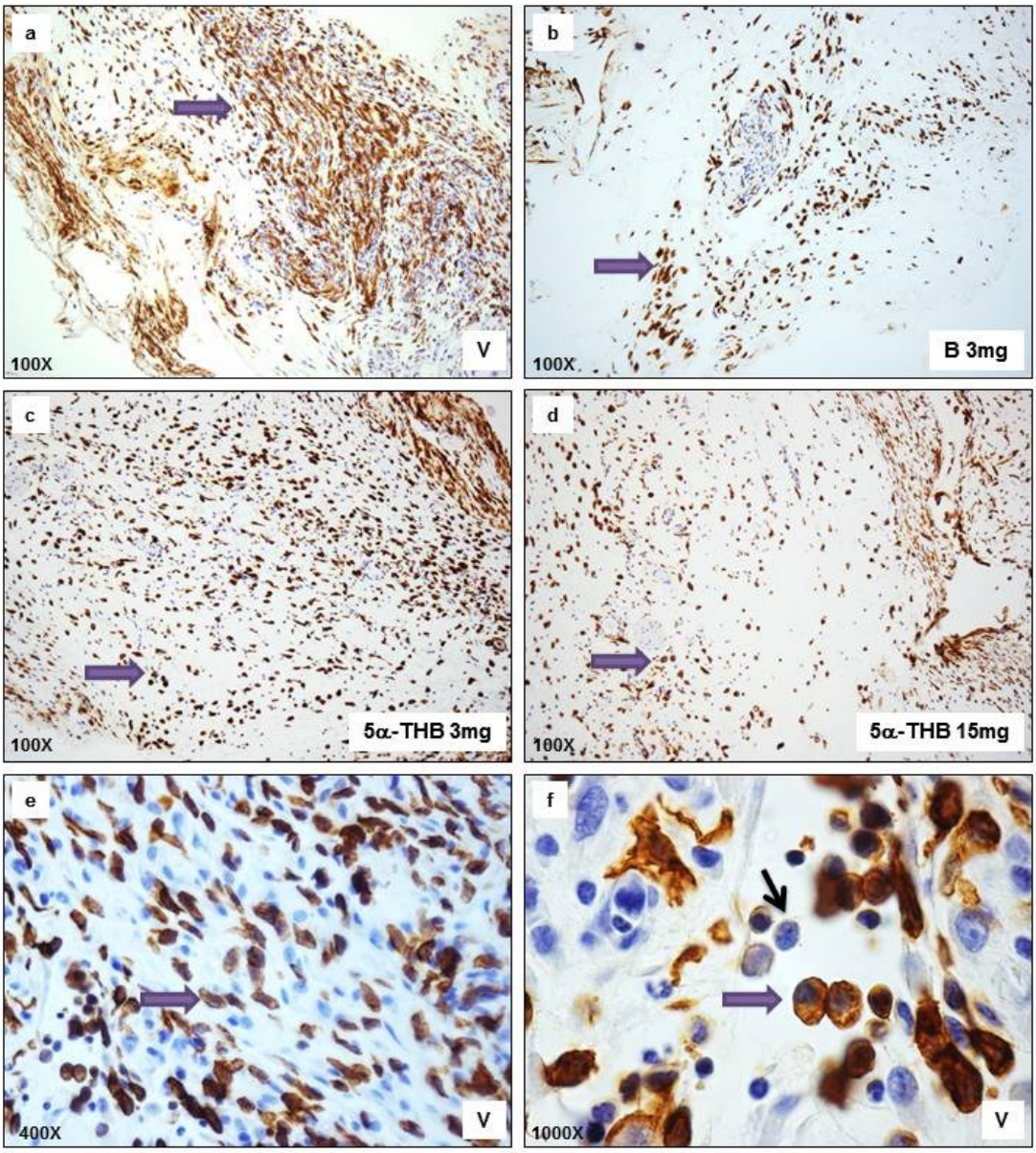

Figure 5.12 5α-Tetrahydrocorticosterone (5α-THB, 15 mg) and corticosterone (B) appeared to reduce the abundance of cells positive for the marker F4/80. Representative images of sponges stained for the marker expressed by macrophages F4/80. V = control group. Magnification = 100X (a)-(d); 200X (e), 400X (f). Purple arrows point to cells positive for the marker; black arrow in (f) indicates a negative cell.

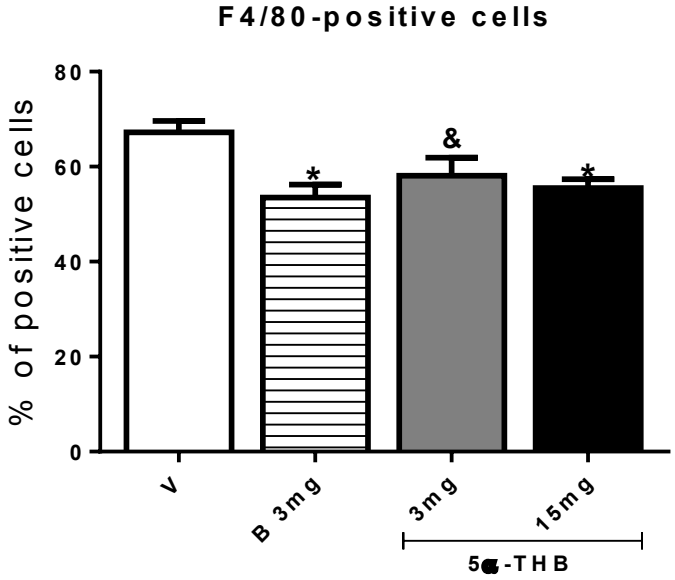

Figure 5.13 5α-Tetrahydrocorticosterone (5α-THB, 15 mg) and corticosterone (B) reduced the proportion of macrophages to a similar extent. Quantification of the proportion of F4/80-positive cells in different experimental groups; V = control group. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. * = $p < 0.05$, & = $0.1 < p < 0.05$ vs V; n = 8-12 mice/group.

5.3.2.4 Effects of 5α-THB on the abundance of transcripts of a panel of genes

The effects of the administration of B and 5α-THB on the abundance of transcripts of genes involved in the formation, maturation and established of a new vascular bed, and in inflammation and signalling, were investigated.

5.3.2.4.1 Genes involved in the remodelling of the vasculature

Transcripts of the genes *Vegf-α*, *Vegfr-2*, *Pecam-1* (also known as CD31), *Cdh5* (Cadherin 5 or VE-cadherin), *Pdgf-β*, *Thsb-1*, *Thsb-2*, *Vcam-1*, *Icam-1*, *E-selectin*, and *Acta2* (αSMA*)* were detectable in all samples (Figures 5.14 and 5.15). Administration of B (3 mg) decreased the abundance of mRNAs of *Vegfr-2*, *Pecam-1* (CD31), *Cdh5* (Figure 5.14 a-c), *Icam-1*, *E-selectin* and *Acta2* (Figure 5.15 d-f), while it increased mRNAs of *Vegf-α* and *Vcam-1* (Figure 5.14 a and 5.15 c) compared with the control sponges. Compared with the same group, 5α-THB had more limited effects as at 3 mg it decreased transcripts only of *Thsb-2* (Figure 5.15 b), and at 15 mg of *Pecam-1* (Figure 5.14 c).

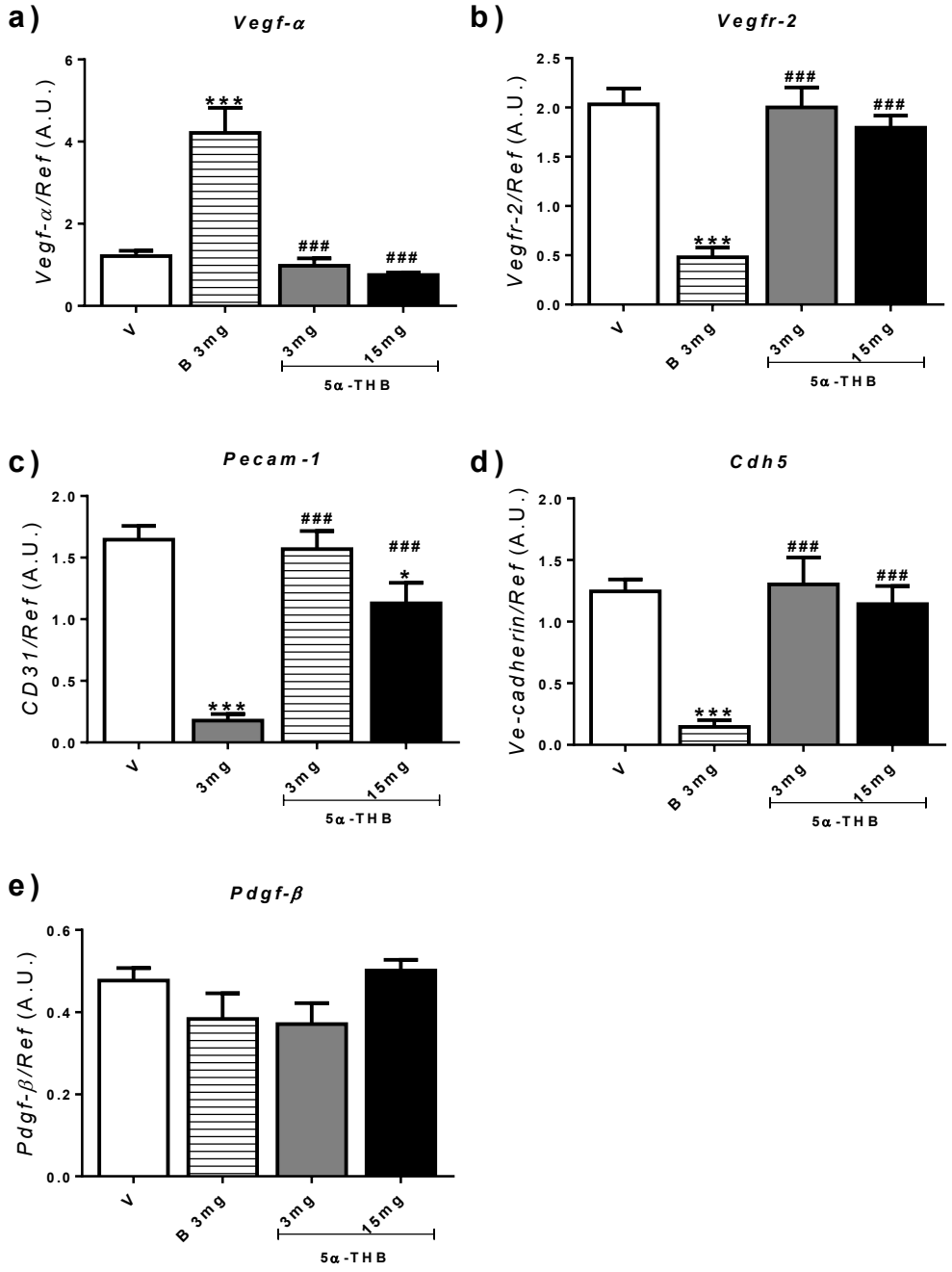

Figure 5.14 5α-Tetrahydrocorticosterone (5α-THB) had limited effects on the abundance of transcripts of genes encoding endothelial molecules. Real-time PCR analysis of transcripts of (a) *Vegfα*, (b) *Vegfr-2*, (c) *Pecam-1*, (d) *Cdh5* (Cadherin 5 or VE-cadherin) and (e) *Pdgf-β*. V = control sponges; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; *** = $p < 0.0001$, * = $p < 0.05$ vs V; ### = $p < 0.0001$ vs B 3mg; n=8-12/group. A.U. = arbitrary unit.

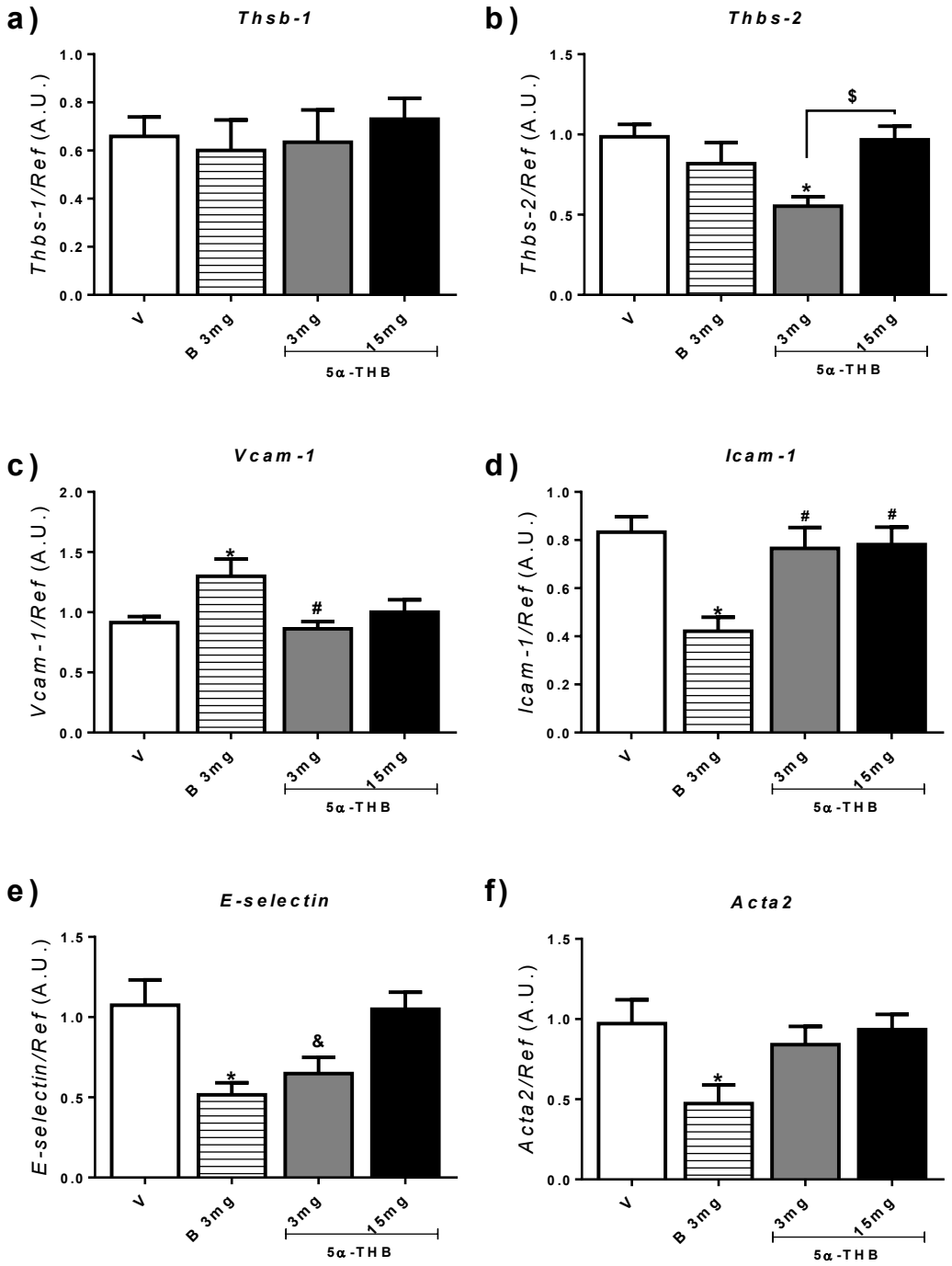

Figure 5.15 5α-Tetrahydrocorticosterone (5α-THB) had more limited effects on the abundance of transcripts of genes involved in angiogenesis. Real-time PCR analysis of transcripts of (a) *Thbs-1*, (b) *Thbs-2*, (c) *Vcam-1*, (d) *Icam-1*, (e) *E-selectin* and (f) *Acta2*. V = control sponges; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; * = $p < 0.05$, & = $0.05 < p < 0.1$ vs V; # = $p < 0.05$ vs B 3 mg; $ = $p < 0.05$; n=8-12/group. A.U. = arbitrary unit.

5.3.2.4.2 Genes involved in inflammation and signalling

Analysis of transcripts of genes with a role in the inflammatory process and in intracellular signalling showed that while B (3 mg) had a broad effect and decreased the abundance of mRNAs of *Tnfα*, *Il1β*, *Infγ* and *Nos3* (also known as endothelial NOS or eNOS) (Figure 5.16 a, c, d, f) compared with the control sponges, the administration of 5α-THB did not decrease any of them, while at 3 mg it increased the abundance of mRNAs of *Mcp1* (Figure 5.16 e).

The analysis was extended also to genes encoding the enzyme responsible for the production of 5α-THB from B, *Srd5a1*, and the two receptors known to be targeted by glucocorticoids, *Nr3c1* (GR) and *Nr3c2* (MR). The investigation showed that B increased the abundance of transcripts of the former and decreased those of *Nr3c1* and *Nr3c2* (Figure 5.17), while 5α-THB did not affect the abundance of any of them.

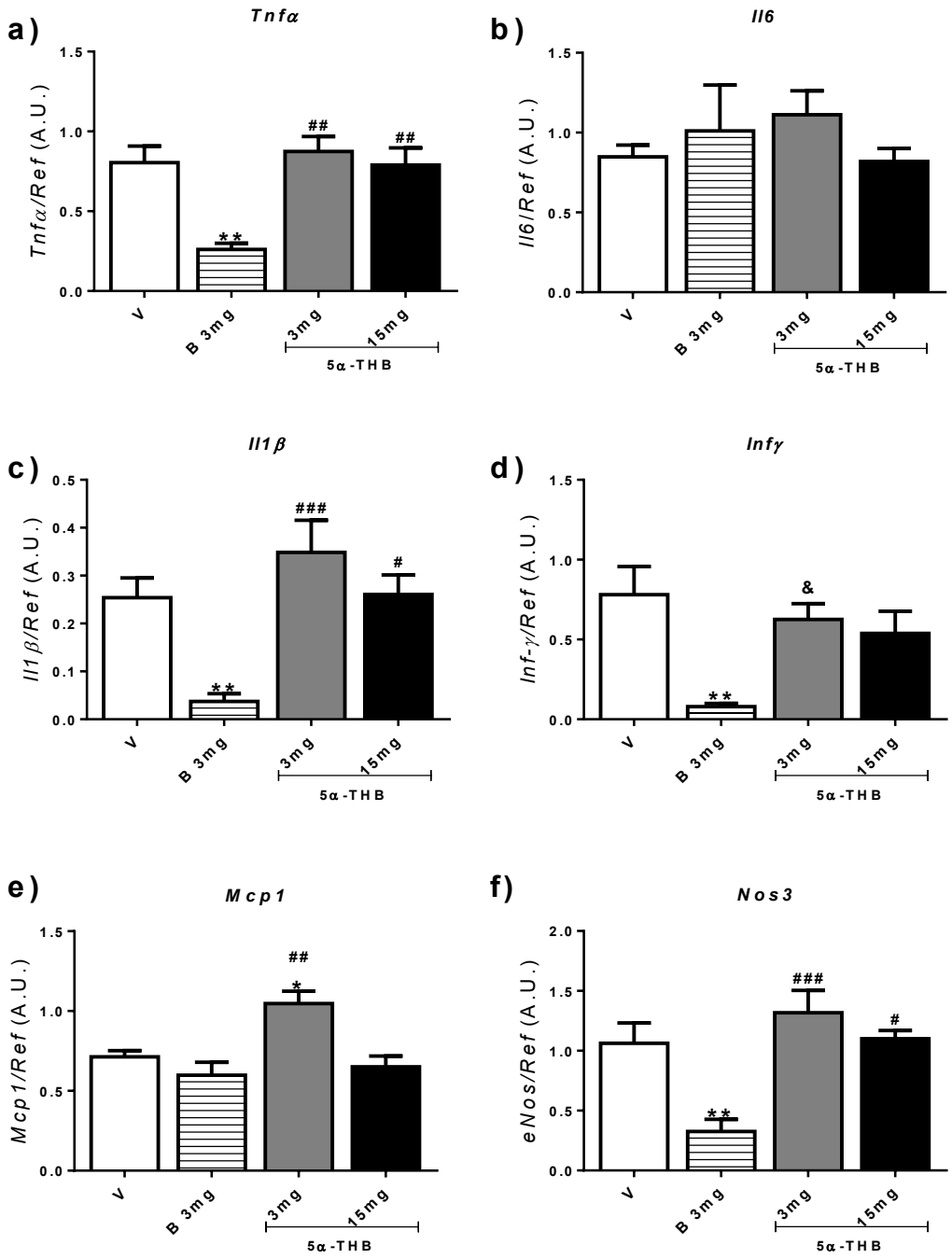

Figure 5.16 5α-Tetrahydrocorticosterone (5α-THB) had limited effects on the abundance of mRNAs of genes with a role in inflammation and signalling. Real-time analysis of transcripts of (a) *Tnfα*, (b) *Il6*, (c) *Il1β*, (d) *Infγ*, (e) *Mcp1* and (f) *Nos3*. V = control sponges; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; ** = $p < 0.001$, * = $p < 0.05$ vs V; ### = $p < 0.0001$, ## = $p < 0.001$, # = $p < 0.05$ vs B 3 mg; n=8-12/group. A.U. = arbitrary unit.

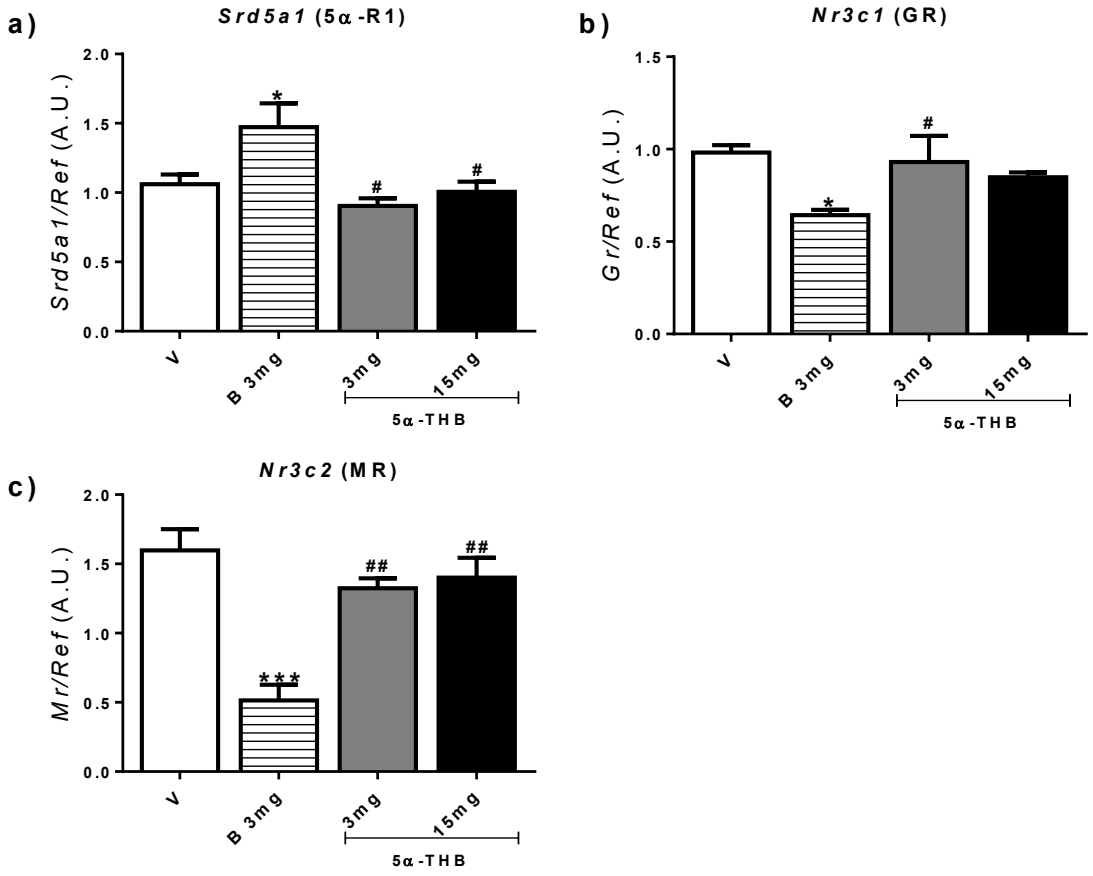

Figure 5.17 5α-Tetrahydrocorticosterone (5α-THB) did not affect the abundance of transcripts of *Srd5a1*, *Nr3c1* and *Nr3c2*. Real-time analysis of transcripts of (a) *Srd5a1* (encoding 5α-reductase type 1 = 5α-R1), (b) *Nr3c1* (GR) and (c) *Nr3c2* (MR). V = control sponges; Ref = average of two housekeeping genes. Data were analysed with one-way ANOVA followed by Tukey's post-hoc test; *** = $p < 0.0001$, * = $p < 0.05$ vs V; ## = $p < 0.001$, # = $p < 0.05$ vs B 3 mg; n=8-12/group. A.U. = arbitrary unit.

5.3.2.4.3 Genes involved in the remodelling of the extracellular matrix

Analysis of the effects of steroids on genes that control the remodelling of the extracellular matrix revealed that the administration of B (3 mg) decreased the abundance of transcripts of *Mmp10* (Figure 5.18 c) compared with the control sponges. 5α-THB had no effect on any of the genes analysed; however, at 15 mg the decrease seen in the amount of mRNAs of *Timp2* showed a statistical trend (Figure 5.18 d).

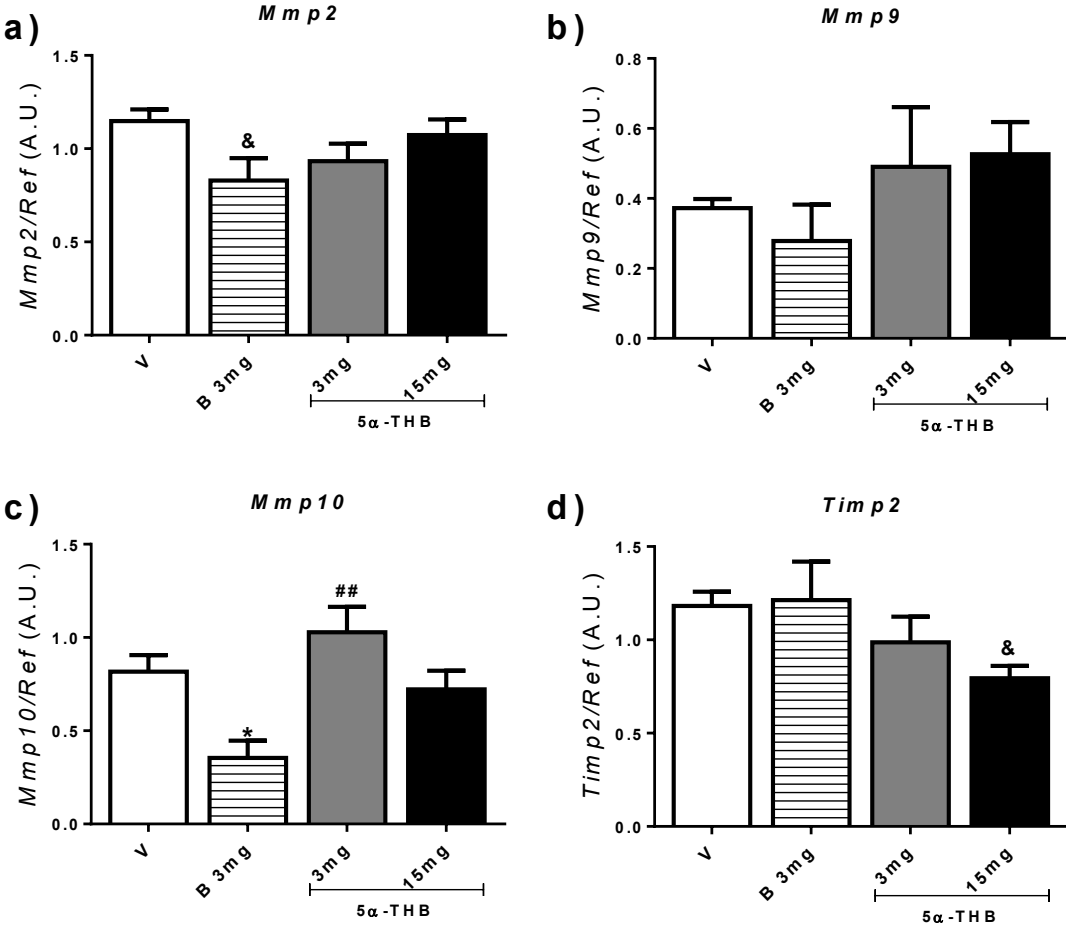

Figure 5.18 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) had limited effects on genes controlling the composition of the extracellular matrix and migration of cells. Real-time analysis of transcripts of (a) *Mmp2*, (b) *Mmp9*, (c) *Mmp10* and (d) *Timp2*. V = control sponges; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; * = $p < 0.05$, & = $0.05 < p < 0.1$ vs V; ## = $p < 0.05$ vs B 3mg; n=8-12/group. A.U. = arbitrary unit.

Analysis of genes encoding different isoforms of the structural molecule collagen showed that the administration of B (3 mg) decreased the abundance of transcripts of all of them (Figure 5.19 a-d) compared with the control sponges, while 5α-THB, at 3 and 15 mg, lowered transcripts only of *Col1a2* (Figure 5.19 b). A trend for 5α-THB, at both concentrations, was present for *Col31a* (Figure 5.19 c).

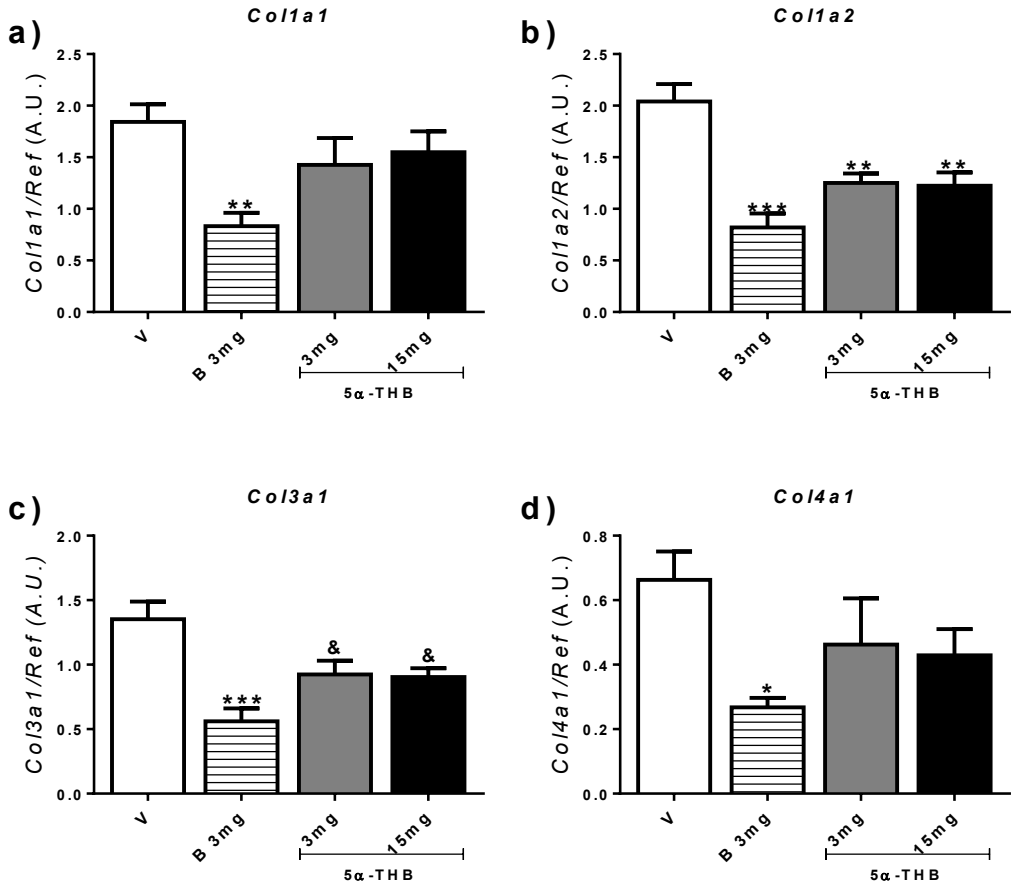

Figure 5.19 5α-Tetrahydrocorticosterone (5α-THB) affected the transcripts of genes encoding different isoforms of collagen to a lesser extent than corticosterone (B). Real-time analysis of transcripts of (a) *Col1a1*, (b) *Col1a2*, (c) *Col3a1* and (d) *Col4a1*. V = control sponges; Ref = average of two housekeeping genes. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$, & = $0.1 < p < 0.05$ vs V; n=8-12/group. A.U. = arbitrary unit.

5.3.2.5 Effects of 5α-THB on the abundance of collagen in sponges

The data obtained with the real-time PCR analysis prompted us to investigate the abundance of collagen in sponges from the different experimental groups through histological analysis. This revealed that administration of B (3 mg) reduced the abundance of collagen fibres compared with the control group (Figure 5.20 a-b and 5.21). In contrast, the treatment with 5α-THB did not affect the content of collagen (Figure 5.20 c-d and 5.21).

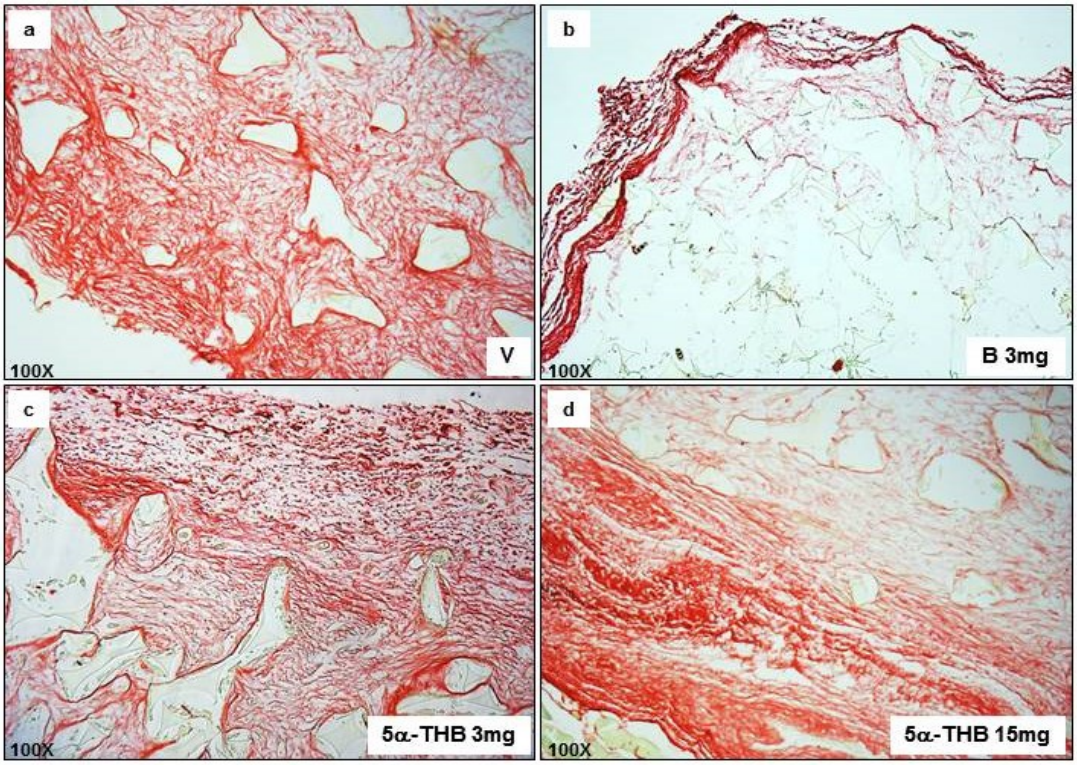

Figure 5.20 5α-Tetrahydrocorticosterone (5α-THB) did not appear to reduce the abundance of collagen in sponges in contrast to corticosterone (B). Representative images of sponges stained for collagen fibres with picrosirius red staining and implanted either with (a) vehicle pellet, (b) B (3 mg), (c) 5α-THB 3 mg or (d) 5α-THB 15 mg. Magnification = 100X.

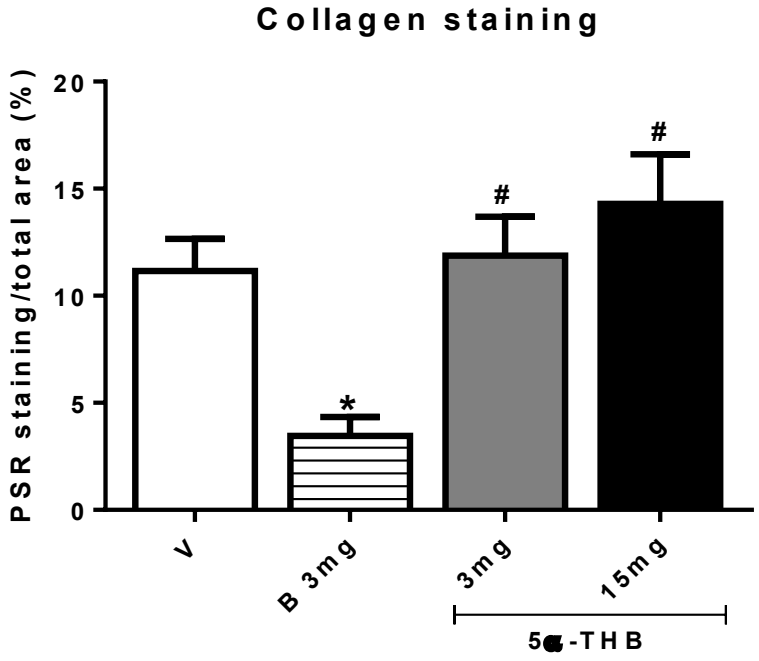

Figure 5.21 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not reduce the abundance of collagen in sponges. Quantification of the intensity of 3,3'-diaminobenzidine (DAB) staining in sponges from the different experimental groups. V = control group. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test. * = $p < 0.05$ vs V; # = $p < 0.05$ vs B 3mg; n = 8-9 mice/group.

5.4 Discussion

In this chapter an *in vivo* model of angiogenesis in mice was established and employed for the investigation of the effects of 5α-THB on the formation of new vessels and on inflammatory pathways/responses. The main finding was that 5α-THB, when given at equipotent doses to B for the reduction of macrophage infiltration, inhibited angiogenesis to a lesser extent than its precursor, indicating a dissociated behaviour. Furthermore, while B had systemic effects, in that it decreased adrenal gland weights, 5α-THB did not. The analysis of different aspects of the angiogenic processes revealed also that, while B decreased the abundance of cells expressing the endothelial marker CD31 (also known as PECAM-1) and of cells positive for the smooth muscle marker αSMA, 5α-THB only decreased the former. The investigation of the effects of the steroidal treatments on the abundance of transcripts of a panel of genes showed a substantial difference between the two glucocorticoids: B affected a variety of transcripts; by contrast, 5α-THB had limited effects. In particular, whereas B strongly decreased the transcripts abundance of genes encoding different isoforms of collagen, 5α-THB only reduced that of one isoform. Histological analysis showed that this translated to a lower amount of collagen fibres in sponges treated with B, but not with 5α-THB.

In 1987, the model of subcutaneous sponge implantation in rats was first introduced in order to study angiogenesis (Andrade, Fan et al. 1987). Since then, a range of bio- and non-biodegradable materials to use as matrix for the growth of new vessels has been tested. Some of the materials employed as implants included steel mesh chambers (Schilling, Joel et al. 1959), synthetic sponge matrix (polyvinyl alcohol, polyester or polyurethane) and Matrigel plugs (Kleinman, Graf et al. 1987). For the experiments presented in this chapter, polyurethane sponges were used as this method was already established in my supervisors' laboratory (Small, Hadoke et al. 2005), having being adapted and refined from literature that used mice (Hague, MacKenzie et al. 2002; Illanes, Dabancens et al. 2002). Furthermore, it is inexpensive, and the number of vessels can be easily and reproducibly quantified by employing simple histological techniques.

Subcutaneous implants constitute the framework in which the angiogenic process, working in concert with the host inflammatory response, can take place and be analysed (Leibovich, Polverini et al. 1987). Upon macroscopic analysis, the appearance of the "vehicle" sponges was consistent with the growth of fibrous tissue around the implant forming a capsule, and the formation of a vascular bed. Microscopic analysis was consistent with previous reports in the literature (Xavier, Amaral et al. 2010) in that the sponge matrix was filled with fibrovascular tissue containing vessels of different calibres, cells and a fibrous stroma.

This *in vivo* model has been widely employed for the study of the effects of a diverse range of compounds on different aspects of the host response to implants. Different methods for the administration of substances have been investigated (Hori, Hu et al. 1996; Hague, MacKenzie et al. 2002; Illanes, Dabancens et al. 2002). Previous work used silastic pellets to deliver glucocorticoids, showing that the release takes place at a controlled rate for three weeks (Cleasby, Livingstone et al. 2003). Subsequent studies applied this method on the sponge model of angiogenesis with successful results (Small, Hadoke et al. 2005), and therefore the same route of delivery was used in this chapter. The dose of B chosen reflected the fact that optimisation studies performed earlier in my supervisors' laboratory revealed that a higher dose of B of 25 mg caused systemic side effects (Small 2006). When this dose was reduced by approximately 10 times (2.5-3 mg), the steroid did not affect organs such as the thymus and adrenal glands, therefore, it was decided to use a concentration of B of 3 mg. In initial experiments, 5α-THB was used at the same concentration as B. The results showed a very small effect of the 5α-reduced steroid on vessel count; as a consequence, since in the previous chapter significant effects on inflammation were robustly obtained with a dose of 5α-THB five times higher than B, it was decided to administer 15 mg of the compound.

The results shown here are in contrast to the abovementioned optimisation studies in that they show an influence of B on adrenal gland weights, indicating the occurrence of systemic effects. In physiological conditions, the HPA axis maintains within certain values the concentration of GCs in the circulation by sensing their abundance

in the blood (Keller-Wood and Dallman 1984). The concentration of B presented in this chapter was within the normal range for C57BL/6 mice (Kotelevtsev, Holmes et al. 1997; Livingstone, Di Rollo et al. 2014). The fact that adrenal gland weights were reduced in animals treated with B suggests that these normal levels were being maintained by the regulatory action of the HPA axis. In effect, if exogenous GCs are given to mice, this would reduce the production from the adrenal glands, and induce atrophy in the long-term. Another result suggesting systemic effects induce by B is the trend towards a significant difference between the number of vessels in control contra-lateral implants of mice receiving B and control sponges of untreated mice. The use of lower concentrations of B could solve this issue. Regarding 5α-THB, in previous studies, systemic infusion by mini-pump did not cause systemic effects (Yang, Nixon et al. 2011; Livingstone, Sykes et al. 2014), and this chapter suggests that this is the case also in the sponge model. However, the analysis of angiogenesis in samples stained with H&E indicated a possible effect of the steroid on the contra-lateral control sponges; therefore, a leakage of 5α-THB cannot be excluded. To investigate this, its concentration would have to be quantified both in sponges and in plasma through mass spectroscopy analysis. As a consequence of these observations, and since the aim of the present study was to investigate the localised and direct effects of steroids, the decision was to consider as control only the sponges from mice not receiving any treatment.

The present study confirmed the anti-angiogenic properties of B and, for the first time, showed that its metabolite, 5α-THB, possesses this quality as well, adding another angiostatic steroid to the list discovered many years ago (Folkman and Ingber 1987). The effects of B given at 3 mg were greater than would have been expected using a dose equal to the EC_{50}, indeed the reduction in the abundance of vessels was approximately 85%; 5α-THB administered at 15 mg caused instead a reduction of 55% which is in accordance with a concentration representing the EC_{50} in this model. Ideally, titration experiments with the two steroids should be performed in order to find/verify the EC_{50} in this model.

During sprouting angiogenesis, endothelial cells lead the formation of new vessels, but in order for the vessels to become functional, they must then recruit other cell types, comprising pericytes and smooth muscle cells. Endothelial and non-endothelial cells can be differentially localised by immunostaining of specific antigens, such as CD31, also known as Pecam-1, and αSMA, or alpha smooth muscle actin. CD31 is a trans-membrane protein expressed by early and mature endothelial cells, primarily concentrated at the junctions between adjacent cells. It is also expressed to a lesser extent by platelets and leukocytes, and plays a crucial role in the trans-endothelial migration of the latter (Jackson 2003). The protein αSMA is found in pericytes and smooth muscle cells, and confers upon them the ability to contract (Bergers and Song 2005). The results presented in this chapter showed that the absolute number of vessels identified with immunostaining against CD31 in the control sponges was higher than the number calculated by using H&E staining. This is likely due to the difference in the sensitivity of the two techniques. Tubular structures, also of very limited dimensions, were clearly visible in sections immunostained for CD31; this allowed for their inclusion in the analysis when they were probably overlooked in sections stained with H&E where the only vessels counted were those clearly containing erythrocytes, to avoid erroneous evaluation. The absolute number of vessels positive for αSMA in control sponges was lower than the number of CD31-positive vessels. This was to be expected, as the presence of αSMA suggests that some of the new vessels have matured and become coated with pericytes or smooth muscle cells, making them a subset of the total amount of vessels. On a technical note, it is worth mentioning that the marker αSMA has been shown to be occasionally expressed also by fibroblasts (Okada, Ban et al. 2000; Inoue, Plieth et al. 2005); the microscopic observation of sponges showed that the staining was not only localised on vessels, but it was also scattered among the stroma surrounding them. This signal may localise fibroblasts, since they are the main producer of fibrous tissue, which can be found in abundance in sponges. To reduce the likelihood that non-specific signal was included in the analysis, only tubular structure characterised by the presence of a lumen, were counted. This was the case also when quantifying the vessels positive for CD31 staining.

Both B and 5α-THB reduced the number of vessels positive for CD31 compared with the control sponges, but only B significantly decreased the abundance of vessels expressing αSMA. When the reduction in CD31-positive vessels was analysed as percentage of the control group, the effects of the steroids were found to be more potent than the effects revealed by the analysis of sections stained with H&E; furthermore, the effects of B (3 mg) and 5α-THB given at 15 mg were comparable, indicating that 5α-THB was five times less potent than B, as found in the dermatitis model (Chapter 3). These observations indicate that because the analysis of sponges stained with H&E was restricted to vessels clearly containing erythrocytes (and therefore functional vessels), as mentioned above, the potency of 5α-THB was initially underestimated. These results point also to an interesting difference between B and 5α-THB, indicating that the former may affect both the initiation and maturation phases of angiogenesis, while the latter seems to affect mainly the initial steps.

Angiogenesis is a process dependent on more than one cellular component, as already mentioned. Fibroblasts, through the synthesis and maintenance of the extracellular matrix, form a stroma that supports old and newly formed vessels; but these cells have also more "active" roles; for instance, it has been recognised that they secrete factors which are required for lumen formation (Newman, Nakatsu et al. 2011). On the other hand, cells from the inflammatory system, in particular monocytes/macrophages, are central players in determining the initiation and the development of the angiogenic process as they are a source of pro-angiogenic cytokines such as VEGF and IL-8 (Sunderkotter, Nikolic et al. 2004). For these reasons, we have analysed the abundance of total cells infiltrating the sponges and, since 5α-THB has been shown to be anti-inflammatory in Chapters 3 and 4, also the proportion of macrophages. B and 5α-THB (15 mg) decreased the total cell number to a similar extent. This investigation did not discern between the different types of cells being counted, and the tissue found in the implants is formed of a variety of cells (Araujo, Rocha et al. 2010), with some of them being precursors of pericytes, endothelial cells or smooth muscle cells. A decrease in the cellularity of the sponges can, therefore, be attributed to the decrease of any of these different populations.

Microscopic analysis of sponges containing B showed that the density of the fibrovascular tissue was lower than that of control sponges. This was seen also for sponges containing 5α-THB at 15 mg, even though to a lesser degree. This would suggest that perhaps the two steroids reduced the recruitment of cells responsible for the deposition of extracellular matrix components.

The inflammatory response is characterised by the recruitment of circulating inflammatory cells at the site of interest, such as neutrophils and monocytes. Neutrophils are the first type of cells to migrate to the tissue, and gradually they leave space for monocytes, which, once activated, differentiate into macrophages (Moura, Lima et al. 2011). Once at the site of inflammation, macrophages proliferate and, together with resident cells and other leukocytes, release mediators that attract other cell types such as fibroblasts and smooth muscle cells to begin a fibro-proliferative phase. All this of course applies also to the inflammatory process taking place in the sponges; because they were retrieved from mice after 20 days *in situ*, it was evaluated that after such a period of time the main inflammatory cell types present would have been macrophages, and therefore decided to analyse this population. The marker F4/80 was chosen to stain macrophages because it has been shown to be the clearest marker for macrophages. Others, such as Mac-1, Mac-2 and CD68, have been found also in fibroblasts isolated from fibrotic tissues (Inoue, Plieth et al. 2005), whereas fibroblasts in culture did not react to staining with antibodies against F4/80 (Austyn and Gordon 1981). The proportion of macrophages was decreased to a similar extent by treatment with B and 5α-THB, at 15 mg, compared with control sponges. This effect could explain, at least in part, the reduction in the total number of cells, and the presence of a less dense stroma, as macrophages have been shown to promote fibrosis (Mosser and Edwards 2008; Mosser and Zhang 2008).

Angiogenesis is a multi-step process that depends on many different mediators, released by a variety of cell types. Any agents that interfere with this process may do so by influencing any of the players involved, be they cellular or molecular. Having investigated how steroids affect some of the cellular components, next we focused on

some of the genes encoding molecules implicated in angiogenesis. Some of the most crucial factors during angiogenesis belong to the VEGF family, being potent pro-angiogenic factors shown to stimulate the proliferation of endothelial cells and their motility (Connolly, Heuvelman et al. 1989; Gerhardt, Golding et al. 2003). The members of this family exert their actions through two tyrosine kinase receptors, VEGFR1 and VEGFR2, with the latter being responsible for the majority of the effects of VEGFs on endothelial cells (Zhang, Fang et al. 2004). The expression of VEGFs and VEGFR2 seem to be correlated temporally and spatially with the proliferation of new vessels during the process of wound healing (Zhang, Fang et al. 2004). One member of this family in particular plays a central role in angiogenesis, VEGFα (Hoeben, Landuyt et al. 2004). The increase in the abundance of transcripts of *Vegfα* in sponges containing B may seem a paradox, since this is a strong stimulus for endothelial cell proliferation and migration, and is considered to be a marker of angiogenesis (Xavier, Amaral et al. 2010); in fact, GCs have been shown to reduce the production of VEGF and the abundance of mRNAs in human smooth muscle cells (Nauck, Roth et al. 1997; Wen, Liu et al. 2003). Furthermore, B decreased the amount of transcripts of *Vegf* in endothelial cells isolated from microvessels of rats (Shikatani, Trifonova et al. 2012). However, some research reported increased levels of VEGF in the cerebral cortex of mice treated with B (Howell, Kutiyanawalla et al. 2011). The increase in transcripts seen here may be seen as a compensatory mechanism to overcome the marked decrease in vessel growth caused by the steroid. The lack of a vascular bed reduces the oxygen supply to the tissue, and this in turn is a strong inducer of angiogenesis, through the production of the hypoxia-inducible factor (HIF) (Pugh and Ratcliffe 2003). The expression of VEGF itself is induced in response to hypoxia (Conway, Collen et al. 2001). GCs have been reported to modulate the expression of HIF, though with contrasting results (Klekamp, Jarzecka et al. 1997; Fischer, Renz et al. 2001; Yossuck, Yan et al. 2001; Michalopoulos, Bowen et al. 2003). It would be interesting to measure the amount of VEGFα in sponges (e.g. by ELISA), to determine whether it correlates with the variations seen in mRNAs; in addition, the expression of HIF in response to steroids could be

studied, as this may point to a mechanism of why B increased the amount of transcripts of *Vegfα*.

The reduction of the abundance of mRNAs of the gene encoding the receptor VEGFR2 was to be expected since it correlates with inhibition of angiogenesis. This phenomenon, through the promotion of a feedback mechanism, may also supply a partial explanation for the increase in transcripts of *Vegfα*. This has been seen *in vivo* in cerebral cortex of mice treated with B (Howell, Kutiyanawalla et al. 2011). Another GC, dexamethasone, has been shown to reduce the expression of VEGFR2 *in vivo* in mice in models of wound healing and allergic dermatitis (Zhang, Fang et al. 2004), and in a murine model of melanoma, conjugated dexamethasone gave similar results (Sau and Banerjee 2014).

During the first phases of angiogenesis, endothelial cells go through a complex rearrangement process in space and time; they must proliferate, migrate and give rise to new tubular structures. During maturation of the newly assembled vessels, the endothelial cells must tighten their interactions to form a controlled and functional barrier. Intercellular junction remodelling plays a central role in both these processes switching between an "inactive" and an "active" mode constantly (Bentley, Franco et al. 2014). PECAM-1 and VE-cadherin are expressed by endothelial cells, and both are important constituents of intercellular junctions. PECAM-1 has been shown to play an important role in the initial formation and stabilisation of cell-cell contact between endothelial cells (Jackson 2003), and VE-cadherin has been found to be pivotal in determining the dynamics of cell rearrangement, making possible the formation of vessels and maintaining their integrity once they are fully formed, so influencing greatly vascular permeability (Goddard and Iruela-Arispe 2013; Bentley, Franco et al. 2014). The reduction of the abundance of transcripts of *Pecam-1* both with B and 5α-THB (15 mg), provided a causal mechanism for the results obtained with immunostaining analysis, and suggest that the two steroids may be reducing the number of vessels by disrupting the dynamics of cell rearrangement. PECAM-1 is a molecule expressed not only in endothelial cells, in effect it is found also in leukocytes and platelets, therefore playing an important role also during the trans-

migration of leukocytes and regulating platelet function (Jackson 2003). As a consequence, the real-time data presented in this chapter may not be only representative of the effects of steroids on a single cell population. The analysis of transcripts of *VE-cadherin* showed no effect of 5α-THB, but a great decrease induced by B. VE-cadherin is a calcium-dependent protein which, once activated, triggers different pathways which signal to the actin cytoskeleton (Goddard and Iruela-Arispe 2013). While B may be disrupting these pathways, including calcium-related ones, 5α-THB may be acting in a more selective manner. The results on the two junction molecules PECAM-1 and VE-cadherin may seem in contrast to the literature, which describes GCs as agents able to tighten intercellular connection, increasing the barrier function of the endothelium. For instance, dexamethasone has been shown to increase expression of VE-cadherin (Blecharz, Drenckhahn et al. 2008), and GCs strengthen the endothelial barrier, most of all at the level of the blood-brain barrier, in conditions characterised by neuroinflammation (Salvador, Shityakov et al. 2014). However, the sponge model presented here was different in that it investigated *de novo* angiogenesis instead of the effects of steroids on an already established vascular bed. Another result in accordance with immunostaining analysis, and providing a molecular mechanism, was the reduction induced by B, but not by 5α-THB, of the abundance of transcripts of *Acta2*, encoding αSMA.

Other genes that were found altered by B, but not 5α-THB, were *Vcam-1*, *Icam-1* and *E-selectin*. These proteins are important in the regulation of vessel permeability during inflammation in that they control extravasation of soluble factors and immune cells (Frank PG 2008). Studies showed that GCs influence the expression of these molecules, and this would explain in part their anti-inflammatory properties (Cronstein, Kimmel et al. 1992; Aziz and Wakefield 1996). However, some other studies have found no effect, claiming that the effects of GCs may be dependent on time, dose and experimental model (Dufour, Corsini et al. 1998). In this chapter, the behaviour of B regarding *Icam-1* and *E-selectin* is in accordance with an anti-inflammatory action. Unexpectedly though, the steroid increased transcripts of *Vcam-1*, an important mediator of the recruitment of leukocytes. It is known that

VEGFα promote expression of VCAM-1 (Fearnley, Odell et al. 2014), and since B increased transcript abundance of *Vegfα* this may provide a causal mechanism.

One of the few genes affected by 5α-THB, at 3 mg, was the anti-angiogenic *Thbs-2*. This may explain the weaker effect on angiogenesis of 5α-THB when given at the lower dose. The reasons why the higher concentration of compound did not have the same, or bigger, effect are not clear. It may be that the higher dose affected other molecules and/or processes in such a way that the transcription of *Thbs-2* was ultimately left unchanged. This protein has been found to affect the remodelling of the extracellular matrix in wound healing and during foreign body response (Kyriakides, Zhu et al. 2001); it is, therefore, possible that its down-regulation may not have had direct and detectable repercussions on angiogenesis as measured by us.

When the abundance of transcripts of pro-inflammatory genes with a role in angiogenesis was investigated, B was found to reduce those of *Tnfα*, *Il1β*, *Infγ* and *Nos3* (eNOS); in contrast, 5α-THB had no effects on any of them. The behaviour of B is in accordance with its extensively-described anti-inflammatory properties. Since these are factors that also stimulate angiogenesis (Leibovich, Polverini et al. 1987), the effect of the steroid may explain in part its anti-angiogenic action. eNOS, an enzyme responsible for the production of the vasodilator NO, plays a role in sprouting angiogenesis by promoting vessel dilation, and its expression is stimulated by VEGF (Bir, Xiong et al. 2012). Reduction of transcripts of this gene by B supplies another mechanism through which this steroid may be inhibiting angiogenesis. GCs have been reported to lower expression of eNOS in endothelial cells (Duckles and Miller 2010). The pro-inflammatory molecule INFγ has been shown to promote activation of macrophages through the classical pathway (Bartneck, Peters et al. 2014). The fact that B decreased accumulation of macrophages in sponges may be linked with its effect on INFγ transcription.

Interestingly, 5α-THB at 3 mg increased the abundance of transcripts of *Mcp1*, while B had no effect. MCP1 is a protein that stimulates the recruitment of monocytes in tissues, therefore increasing the abundance of macrophages. Over-expression of

MCP1 has been shown to promote vessel growth and functionality in/of infarcted hearts, presumably by a mechanism linked with increased infiltration of macrophages (Morimoto, Takahashi et al. 2006). 5α-THB did not increase the abundance of macrophages or improve angiogenesis therefore, this result may seem contradictory. This increase may be a reaction to the presence of a constant inflammatory stimulus (the implants), which stimulates the recruitment of cells of the immune system. A similar result was seen also in a study using a model of sponge-induced angiogenesis to test the effects of metformin (Xavier, Amaral et al. 2010), suggesting this may be a real compensatory mechanism. As with the result regarding *Thbs-2*, while the lower dose of 5α-THB affected abundance of mRNAs of *Mcp-1*, the higher dose did not have any effects. Having encountered this phenomenon twice, it is reasonable to believe that it is representative of a real occurrence rather than a chance result. As mentioned above, the higher dose of steroid may be influencing a greater number of factors and processes, probably not yet investigated, which in turn may keep constant the transcription of *Mcp-1*.

Cell adhesion, migration and vessel formation during angiogenesis are processes closely related to the remodelling of the extracellular matrix (ECM). Central players of this remodelling are the metalloproteinase enzymes (MMPs), which promote ECM degradation, and tissue inhibitors of MMPs (TIMPs), which counteract the destructive actions of MMPs (Pepper 2001; Bellon, Martiny et al. 2004; Sottile 2004). It is known that GCs influence the expression of both groups of enzyme (Perretti and Ahluwalia 2000; Pitzalis, Pipitone et al. 2002; Shikatani, Trifonova et al. 2012). For example, decreased expression of MMP2 and increased synthesis of TIMP2 cause by GCs have been reported before (Pross, Farooq et al. 2002; Shikatani, Trifonova et al. 2012). The data presented here show that B only reduced the transcript abundance of *Mmp10*, while 5α-THB had no effect on any of the genes. The enzyme MMP10 is known to degrade the basement membrane, together with MMP3 and MMP9 (Davis 2010), promoting cell migration. If the decrease in transcription induced by B translates into decreased production of MMP10, this would point to reduced ECM degradation with consequent reduction in remodelling, which is so important in angiogenesis. However, the effect of B on the panel of

MMPs tested seemed rather limited, so the functional significance cannot be easy judged. Another important component of the ECM that was investigated was collagen. B reduced the amount of transcripts of all four isoforms studied, while 5α-THB had a more restricted effect. A plethora of studies have shown that GCs induce degradation of the ECM by interfering with production of collagen, and this phenomenon is known to cause some of the side effects of GC therapy, such as skin thinning and impairment of wound healing. Different kinds of collagen are found in different types of extracellular structures, and GCs have been shown to interfere with many of these (Maragoudakis, Sarmonika et al. 1989; Grose, Werner et al. 2002; Oishi, Fu et al. 2002). Staining of collagen fibres in section of sponges revealed that their abundance was reduced in samples treated with B but not with 5α-THB. This may be due to the fact that 5α-THB only affected the abundance of transcripts of one type of collagen. When sponges were analysed microscopically, it was clear that the fibro-vascular matrix of those treated with B was much less compact that the control group. It was also noticed that those treated with the higher dose of 5α-THB showed a looser structure, even though the effect was less pronounced. The results obtained from the staining were therefore slightly unexpected. However, other proteins constitute the ECM, and GCs have been shown to have an effect on some of these. An example comes from a study conducted almost 30 years ago in which GCs were found to decrease, during angiogenesis, two components of the basement membrane, laminin and fibronectin, and this phenomenon was thought to explain their anti-angiogenic properties (Folkman and Ingber 1987). It is possible that 5α-THB, while not affecting the production of collagen, may affect that of other proteins not yet analysed.

The presence of B in the implants increased the abundance of transcripts of *Srd5a1*, while 5α-THB had no effect. This increase indicates that a positive feedback of B on its own metabolism may exist, perhaps in order to decrease it deleterious effects on angiogenesis. In a model of umbilical cord occlusion in the sheep, hypoxia has been shown to increase expression of 5α-R2 in the brain (Nguyen, Ross Young et al. 2004); this may represent a stimulus also for 5α-R1 in sponges treated with B. It is challenging to make any assumptions about which kind of cells are transcribing the

gene in sponges. Some groups found the presence of 5α-reductases in human, rat and murine vessels and monocytes/macrophages, however, they did not confirm the identity of the isoforms (Nixon, Upreti et al. 2011). It has also been shown that fibroblasts express 5α-R1 (Hoppe, Holterhus et al. 2006). All these cell populations are present in the fibrovascular tissues in sponges; as a consequence, more detailed studies may have to be performed to clarify this point, perhaps by isolating the different cell types by FACS analysis.

Previous studies using the sponge model of angiogenesis showed that the angiostatic effects of GCs are dependent on GR (Small, Hadoke et al. 2005; Logie, Ali et al. 2010); the former study also confirmed this *ex vivo*, using the aortic ring model of angiogenesis, and showed that MR did not seem important for the sprouting of vessels in this experimental set-up. At the same time, GCs are known to bind both GR and MR, and some of their effects on the cardiovascular system seem to be dependent on MR. This receptor is expressed by cardiac myocytes, where the absence of the enzyme 11β-HSD2 makes it a target for activation by GCs (Yang and Fuller 2012). Additionally, endothelial cells and vascular smooth muscle cells express MR (Meijer 2002; McCurley, McGraw et al. 2013), and a body of literature exists suggesting a negative influence of the receptor on angiogenesis (Tiberio, Nascimbeni et al. 2013). Interestingly, activation of MR in cell cultures that over-expressed the receptor correlated with decreased expression of VEGFα (Tiberio, Nascimbeni et al. 2013). The effect of B on transcripts of *Nr3c1* (GR) and *Nr3c2* (MR) suggests a functional interaction of the steroid with the receptors; an interaction that may lead also to a mechanism of negative feedback. We could speculate that the reduction in transcripts of *Nr3c2* may account, at least in part, for the increase of *Vegfα* mRNAs, and see this as a compensatory mechanism. This effect may also provide a mechanism through which B inhibited collagen deposition, as it has been shown that MR is involved in the fibrotic response (Meinel, Gekle et al. 2014). The absence of effects with the administration of 5α-THB suggests a couple of possibilities: the two receptors may not be the targets of this steroid in this model, or the action of 5α-THB may be not strong enough to alter the two genes.

As with any analysis that investigates the abundance of transcripts, the results obtained by real-time PCR in this chapter did not provide any information about the abundance of the proteins encoded by the genes. It would be, therefore, very important to extend the analysis by using techniques such as immunostaining, Western blotting and/or ELISA, in order to understand whether the results presented here could have functional repercussions. It is well known that GCs can modify protein abundance by influencing translational mechanisms (Clark 2003), therefore transcript analysis can only give a very partial picture of the potential mechanisms of action of B and 5α-THB, in this and in any model.

In conclusion, 5α-THB, administered at equipotent doses to B for reduction of macrophage infiltration, possessed weaker anti-angiogenic properties. Importantly, 5α-THB affects the abundance of transcripts of a more restricted number of genes in comparison with its precursor. One reason for this behaviour may be that 5α-THB works through a different receptor than B, as suggested by the results presented in Chapter 3 and 4 using the GR antagonist RU486. This needs to be verified in the sponge model with studies aimed to inhibit GR, and possibly MR.

Chapter 6

EFFECTS OF 5α-THB ON GLUCOCORTICOID RECEPTOR SIGNALLING PATHWAYS

Chapter 6: Effects of 5α-THB on glucocorticoid receptor signalling pathways

6.1 Introduction

Classically, the effects of glucocorticoids (GCs) on gene expression have been explained by two main types of GR-dependent mechanisms, trans-activation and trans-repression. The former has been largely associated with the side effects induced by long-term therapy with steroids, while the latter has been suggested as the means through which these molecules deliver their beneficial anti-inflammatory effects. As a consequence, the search for substitute compounds has focussed on finding molecules that work mainly through trans-repressive mechanisms. This, of course, is a simplistic picture because GCs have a much more complex behaviour, which we have begun to discover only very recently. As reductionist as it may be, the simplified division between trans-activation and trans-repression does help to identify new more selective anti-inflammatory compounds. Of course, the initial screenings must subsequently be refined in order to gain a fuller image of the real potential of newly discovered molecules.

In previous chapters, the anti-inflammatory properties of 5α-THB were investigated in *in vivo* and *in vitro* models; in addition, the compound was tested *in vivo* for effects on angiogenesis, a process which GCs are known to inhibit causing some of their most common side effects (i.e. impairment of wound healing). These studies provided a preliminary picture of what molecular mechanisms are influenced by the 5α-reduced steroid, and revealed that the compound has weaker effects than B in many systems and may work through pathways not requiring GR. In the present chapter, the focus will be on delving deeper into these mechanisms and, in particular, in understanding how 5α-THB affects gene expression mediated by trans-activation. This mechanism is also held responsible for some of the anti-inflammatory properties of GCs, thanks to induction of anti-inflammatory proteins; therefore, the present study will provide a fuller picture of the working mechanisms of 5α-THB, and of its potential as a new more selective anti-inflammatory drug. Furthermore, it will shed

light on how a metabolite physiologically produced, and until recently believed inactive, may influence cellular physiology.

A range of *in vitro* experimental approaches will be employed, including analysis of the induction of luciferase reporters and endogenous genes, and of post-translational modification of GR, such as phosphorylation, known to affect transcriptional activity of the receptor.

Hypotheses

- 5α-THB and B differentially affect the phosphorylation state of the glucocorticoid receptor

- 5α-THB and B modulate different profiles of signalling pathways regulated by the glucocorticoid receptor

Objectives

The aims of this chapter were to investigate:

- the profile of phosphorylation of the GR in response to 5α-THB

- the effect of 5α-THB on the expression of GR-driven reporter plasmids and steroid-responsive endogenous genes

6.2 Materials and Methods

6.2.1 General

6.2.1.1 Materials

Materials for general cell culture and experimental treatments were obtained as described in section 2.1.1.1. Steroids, including the GR antagonist RU486, were from Steraloids (Newport, RI, USA). The carcinomic human alveolar cell line A549, and the mouse hepatoma cell line BWTG3, were sourced and maintained in culture as described in sections 2.1.1.2 and 2.1.2.

6.2.1.2 Preparation of steroids

Steroids were dissolved in ethanol to obtain solutions 1000 times more concentrated than the concentration needed in the experiments. This allowed the addition of 1 μL of steroid solutions into each mL of medium at the time of treatment. The solutions were stored at -20 ºC for a maximum of one month before being discarded and replaced with freshly made ones. When needed they were left at RT for 30 min prior to usage.

6.2.1.3 General treatments

Steroids were added directly to the culture medium into each well, at a concentration and for the length of time indicated. Each treatment was always performed in triplicate, and each experiment was repeated between three and eight times as indicated.

6.2.2 A549 cells

6.2.2.1 General treatments

In all experiments, A549 cells ($4x10^5$cells/well) were plated in stripped-FBS medium on 12-well plates; 24 h later, cells were treated in triplicate with steroids in ethanol or ethanol (vehicle as control) for the time and at the concentration indicated.

6.2.2.2 Studies on phosphorylation of GR

6.2.2.2.1 Development of a phosphorylation assay for Ser211

Western blotting was used to investigate phosphorylation of GR. In order to test the responsiveness of the A549 cell line to steroid treatment, cells were incubated either

with 5α-THB, B or vehicle for 1 h. To confirm the identity of the protein detected, GR abundance was suppressed using RNA interference technology.

6.2.2.2.2 Time course of phosphorylation of Ser211

Time-dependent phosphorylation of GR was investigated by Western blotting after incubation of A549 cells with either steroids or vehicle for 1, 5 and 24 h.

6.2.2.2.3 Knockdown of GR by RNA interference (RNAi)

The procedure was performed as described in section 2.1.3.1.

6.2.2.2.4 Western blotting

6.2.2.2.4.1 Materials

General materials were obtained as described in section 2.3.3.1.1. Specific primary antibodies employed were: rabbit polyclonal antibody to GR phosphorylated at serine 211 ($^{P\text{-}Ser211}$GR) from Cell Signalling Technology (Hertfordshire, UK); rabbit polyclonal antibody to GR and mouse monoclonal antibody to β-tubulin from Insight Biotechnology (Wembley, Middlesex, UK); secondary antibodies were: IRDye 800CW Goat Anti-Rabbit IgG (H+L) and IRDye 800CW Goat Anti-mouse IgG (H+L) from LI-COR Biosciences Ltd (Cambridge, Cambridgeshire, UK). Primary antibodies were diluted as followed: $^{P\text{-}Ser211}$GR 1:1000, Total GR 1:500, β-tubulin 1:1000 in BSA (5% w/v in TBST), and all secondary antibodies were diluted 1:10000 in powder cow's milk (5% w/v in TBST).

6.2.2.2.4.2 Procedures

Protein extraction from A549 cells, quantification and Western blotting analysis were performed as described in sections 2.3.3.1.2, 2.2.3.1.3 and 2.3.3.1.4.

6.2.2.2.4.3 Quantification of protein expression

The abundance of protein was calculated by using the Odyssey® Imaging software (LI-COR biosciences, Cambridge, Cambridgeshire, UK). The abundance of phosphorylated GR was normalised to the total amount of GR. In order to do that, two gels were run at the same time, on the same apparatus, and loaded with the same samples; the quantification was performed as followed: the ratios between total GR and β-tubulin in one gel, and between $^{P\text{-}Ser211}$GR and β-tubulin in the other, were

calculated; subsequently, phosphorylation was expressed as ratio between these two ratios for each treatment. In blots in which $^{P\text{-}Ser211}$GR was detected, two bands were visible at the molecular weight predicted; both bands were quantified. Regarding knockdown experiments, the percentage of knockdown was calculated by considering the ratio for control wells as 100% GR expression.

6.2.2.3 Studies of transcription of endogenous genes

6.2.2.3.1 Effects of 5α-THB on transcription of endogenous genes

Cells ($4x10^5$cells/well) were treated either with vehicle, B (1μM) or 5α-THB (1, 3, 10 μM) for 2 h prior to total RNA extraction (described in section 2.3.1.2).

6.2.2.3.2 Real-time PCR analysis

Production of cDNA and real-time PCR analysis were performed as described in sections 2.3.1.5 and 2.3.1.6 respectively. The details of the human primers and UPL probes employed are given below in table 6.1.

Gene symbol, full name, accession No	Primers sequence	UPL probe
Dusp1 (dual specificity phosphatase 1), NM_004417.3	ttcaacgaggccattgactt	65
	cctggcagtggacaaacac	
Gilz (glucocorticoid-induced leucine zipper), AB025432.1	ccgttaagctggacaacagtg	36
	atggcctgttcgatcttgtt	
Igfbp1 (insulin-like growth factor binding protein 1), NM_000596.2	gccttggctaaactctctacga	12
	ccatgtcaccaacatcaaaaa	
Fkbp51 (FK506-binding protein 51), U71321.1	ggatatacgccaacatgttcaa	15
	ccattgctttattggcctct	

Table 6.1 Sequences of the primers and UPL probe numbers used for real-time PCR in A549 cells. The reverse primer (3' → 5') for each gene is above the separation line, the forward primer (5' → 3') is below.

6.2.2.4 Studies on expression of reporter plasmids

6.2.2.4.1 Transient transfection

6.2.2.4.1.1 Material

General materials were obtained as described in section 2.1.3.2. Two GR-responsive luciferase reporter plasmids were employed: MMTV-LTR-Luc reporter (Mouse Mammary Tumor Virus-Long Terminal Repeat-Luciferase, abbreviated as MMTV-Luc) activated by GR-dimer binding (Lefebvre, Berard et al. 1991; Michailidou, Carter et al. 2008); rPNMT -998/-446-Luc reporter (rat Phenylethanolamine N-Methyltransferase -996/-446 Luciferase, abbreviated as PNMT-Luc) activated by binding of GR multimers (Adams, Meijer et al. 2003; Michailidou, Carter et al. 2008). The plasmids were kindly provided by Prof. K.E. Chapman, Centre for Cardiovascular Science, University of Edinburgh (rPNMT-998/-466 Luc was originally from David Pearce, University of California, Department of Medicine, San Francisco, CA, USA). Both plasmids were originated by cloning the appropriate regulatory sequence of MMTV or PNMT gene into the pGL3-luciferase reporter plasmid. In addition, the following plasmids were utilised: pGEM, plasmid containing inert DNA, as negative control; pSV2-Luciferase, plasmid used as positive control to confirm success of transfection; pGL3-Luciferase, reporter lacking regulatory sequences (promoter/enhancer) used as negative control; pVL342, plasmid containing the sequence encoding the murine GR; pKC275, β-Galactosidase plasmid, used as internal control for transfection efficiency.

The plasmids pGEM and pGL3-Luciferase were purchased from Promega (Hampshire, UK); pVL342 and pKC275 were designed and prepared by Valery Kelly (Prof K.E. Chapman's group), and kindly provided by Dr. K. McInnes along with pSV2-Luciferase. The maps of pVL342 and pSV2-Luciferase can be found in (Michailidou 2007). The cloning and expression of a plasmid encoding mouse GR was first described by (Danielsen, Northrop et al. 1986).

6.2.2.4.1.2 Transfection procedure

The amplification of plasmids and their transfection into A549 cells were performed as described in section 2.1.3.2. The table below summarises the plasmids used with the relative amount of DNA (expressed as μg per each well).

Plasmid (µg)	Transfection						
	pGEM	pSV2	pGL3	+ GR		- GR	
				PMNT	MMTV	PMNT	MMTV
pGEM	2.2	-	-	-	-	-	-
pSV2-luc	-	2.2	-	-	-	-	-
pGL3-basic	-	-	2.2	-	-	-	-
PMNT-Luc	-	-	-	1	-	1.2	-
MMTV-Luc	-	-	-	-	1	-	1.2
pVL342	-	-	-	0.2	0.2	-	-
pKC275	-	-	-	1	1	1	1

Table 6.2 Schematic representation of the plasmids, with relative quantities, transiently transfected in A549 cells.

6.2.2.4.2 Experimental conditions and treatments

6.2.2.4.2.1 Development of an assay to detect expression changes in response to steroids

In order to test that the reporters were responsive to steroids, A549 cells were treated with B (1µM). To analyse whether their activation was GR dependent two approaches were tested: (a) OVER-EXPRESSION OF GR; cells were transfected either with the reporters alone or together with a plasmid coding for murine GR (mGR) and then treated with B, and (b) PHARMACOLOGICAL GR ANTAGONISM; cells were treated with B (1 µM) only or with B (1 µM) + RU486 (0.1 and 1 µM). RU486 powder was dissolved in ethanol to obtain a concentration 1000 times greater than that needed in the experiments, and diluted directly by addition to the cell culture medium in the wells. The solution was freshly prepared prior to each experiment. Treatments were started 1 h before the addition of B.

6.2.2.4.2.2 Effect of 5α-THB on expression of reporter plasmids

Once the model was tested for responsiveness to steroids and GR-dependency of induction, effects of 5α-THB on the expression of the two plasmids were investigated. To this end, concentration-response comparisons between B and 5α-THB were performed.

6.2.2.4.3 Luciferase and β-galactosidase assays

The assays were performed, and data recorded, as described in 2.3.3.2.

6.2.2.4.4 Data analysis

When the luciferase activity of cells transiently transfected with pSV2-luc was 400 times higher than that of pGEM, the transfection was deemed successful. Activity from pGEM was subtracted from all values as a measure of background activity. Quantification of luciferase activity was achieved by calculating the ratio between the values of the chemioluminescence generated by luciferase activity and those recorded during β-galactosidase assay.

6.2.3 Gene transcription studies in BWTG3 cells

6.2.3.1 General treatments

In all experiments, the murine hepatoma cell line BWTG3 cells ($5x10^5$cells/well) were plated in stripped-FBS medium on 12-well plates; 24 h later, cells were treated in triplicate either with steroids in ethanol or ethanol (vehicle as control) for the time and at the concentrations indicated.

6.2.3.2 Development of an assay to detect gene transcription changes in response to steroids

Cells were tested for responsiveness to steroids by investigating the abundance of transcripts of the gene tyrosine aminotransferase (*Tat*) as representative steroid-responsive gene following treatment with dexamethasone (dex) and B for 1, 4 and 6 h.

6.2.3.3 Effects of 5α-THB on endogenous gene expression

Once the abundance of mRNAs for TAT had been tested, the effect of treatment with 5α-THB for 4, 8, 16 and 24 h was investigated. Subsequently, 16 h was taken as the

length of treatment to use in following experiments where the mRNA abundance of other endogenous genes was analysed.

6.2.3.4 Modulation by 5α-THB of B-induced effects

In order to understand if 5α-THB was able to interfere with the effects of B on gene transcription, cells were co-treated with B (1 μM) + 5α-THB (0.01-10 μM) for 16 h.

6.2.3.5 Real-time PCR analysis

Total RNA extraction, cDNA production and real-time PCR analysis was performed as described in section 2.3.1. The details of the murine primers and UPL probes employed are given below in table 6.3.

Gene symbol, full name, accession No	Primers sequence	UPL probe
Gilz (glucocorticoid-induced leucine zipper), NM_001077364.1	tccgttaaactggataacagtgc	49
	tggttcttcacgaggtccat	
Ggt1 (γ-glutamyltransferase 1), NM_008116.2	tcacagcccagattgtgaaa	79
	agcacggtagttgttgaggtc	
Igfbp1 (insulin-like growth factor binding protein 1), NM_008341.4	tggtcagggagcctgtgta	62
	acagcagcctttgcctctt	
Tat (tyrosine aminotransferase), NM_146214.3	ggaggaggtcgcttcctatt	82
	gccactcgtcagaatgacatc	

Table 6.3 Sequences of the primers and UPL probe numbers used for real-time PCR in BWTG3 cells. The reverse primer (3' → 5') for each gene is above the separation line, the forward primer (5' → 3') is below.

6.2.4 Data analysis

6.2.4.1 General

Data were represented as mean ± SEM and analysed as described in section 2.4.

6.2.4.2 Real-time PCR

For quantification of transcripts through real-time PCR, the abundance of each gene was represented in relation to the abundance of a chosen housekeeping gene. The housekeeping gene used for A549 cells was CyclophilinA (*CypA*), and for BWTG3 cells *Tbp*, due to the lack of significant changes in the abundance of transcripts following treatment with steroids. For each sample and each gene studied, the values presented in the graphs were calculated as follow: value sample X/value *CypA or Tbp* sample X.

6.3 Results

6.3.1 Effects of 5α-THB on GR phosphorylation in A549 cells

6.3.1.1 Development of a phosphorylation assay for Ser211

As shown in Figure 6.1 a and b, the cell line A549 was responsive to treatment with B (1 μM) as the steroid increased phosphorylation of GR at Ser211 compared with vehicle-treated cells (basal level) after 1 h of incubation. At this time point, 5α-THB (1 μM) did not increase phosphorylation above basal level.

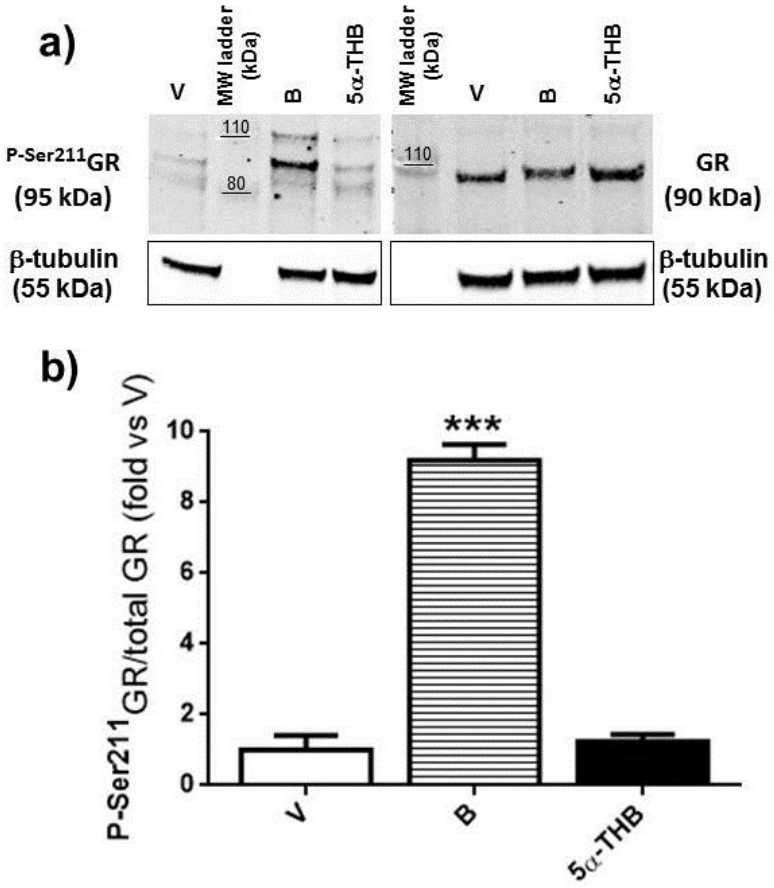

Figure 6.1 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not induce phosphorylation of serine 211 on the glucocorticoid receptor (GR). (a) Representative images of Western blot analysis of total GR (right) and phosphorylated GR ($^{P\text{-}Ser211}$GR, left) in A549 cells treated for 1 hour either with B, 5α-THB (both 1μM) or vehicle (V). (b) Quantitative analysis of GR phosphorylation after normalisation against β-tubulin. Data (mean ± SEM) were analysed by one-way ANOVA with Tukey's post-hoc test; *** $p<0.0001$ vs V; N = 4. MW = molecular weight, kDa = kilo Dalton.

6.3.1.2 GR knock down

GR was knocked down by about 40% compared with control treated cells (Figure 6.2 a). Using this approach, it was confirmed that the correct protein was being studied in experiments of phosphorylation of GR on Ser211 (Figure 6.2 b).

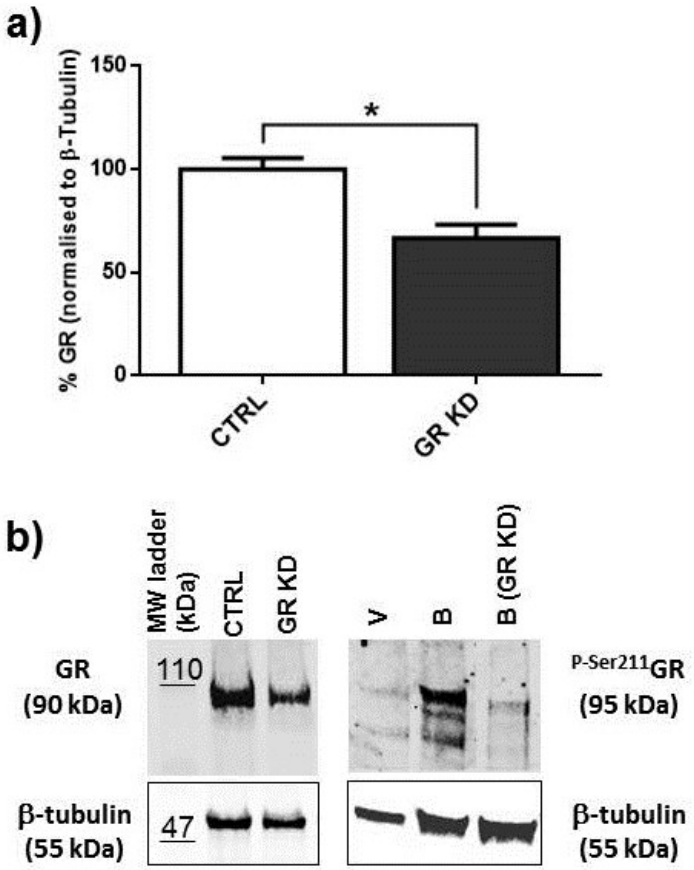

Figure 6.2 **Glucocorticoid receptor (GR) was knocked down through RNA silencing (siRNA).** (a) Quantification of the extent of GR knock down; GR amount was normalised against β-tubulin; CTRL = control, cells treated with vehicle only; N = 3; * = $p < 0.05$ vs CTRL with unpaired t-test. Data are mean ± SEM. (b) Detection of GR (left side) and phosphorylated GR ($^{P\text{-}Ser211}$GR, right side) upon treatment with vehicle (V) or B for 1 hour. MW = molecular weight, kDa = kilo Dalton. CTRL, V and B = cells transfected with mock siRNA; GR KD = cells transfected with siRNA for GR.

6.3.1.3 Time-dependent phosphorylation of GR at Ser211 in response to treatment with 5α-THB

As shown in Figure 6.3, treatment with B of A459 cells increased phosphorylation of GR at Ser211 ($^{P\text{-}Ser211}$GR) compared with vehicle treated cells at every time point studied, with a maximum occurring at 5 h post-treatment and a minimum at 24 h. Addition of 5α-THB increased phosphorylation compared with the vehicle-treated group only at a concentration of 10 μM after 1 h of treatment; this effect was weaker than that of B at the same time point.

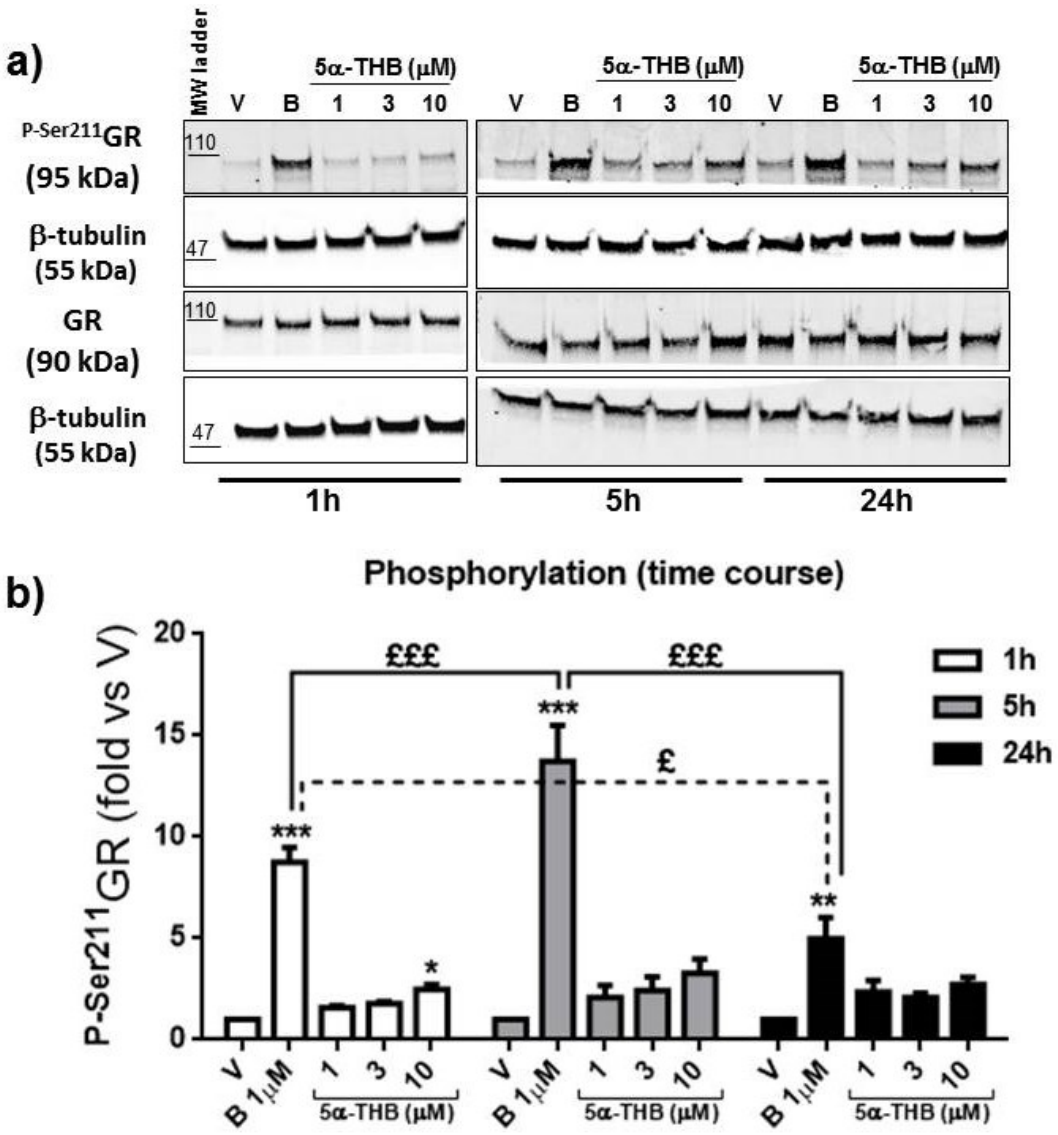

Figure 6.3 5α-Tetrahydrocorticosterone (5α-THB) induced phosphorylation of the glucocorticoid receptor (GR) with a different kinetics compared with corticosterone (B). (a) Representative image of a Western blotting for phosphorylated GR ($^{P\text{-}Ser211}$GR), total GR (GR) and β-tubulin. MW = molecular weight, kDa = kilo Dalton. (b) Quantification of GR phosphorylation performed as described in section 6.2.2.2.4.3. Vehicle group was considered as one fold induction at each time point and steroid-stimulated phosphorylation was calculated accordingly. N = 8 analysed by one-way ANOVA with Tukey's post-hoc test. *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$ vs V; £££ = $p < 0.0001$, £ = $p < 0.05$. Data are mean ± SEM.

6.3.2 Effects of 5α-THB on the abundance of transcripts of endogenous genes in A549 cells

Analysis of the effects of steroidal treatment (2 h) on the abundance of transcripts of selected steroid-responsive gene (Figure 6.4 a-d) revealed that B (1 μM) increased that of *Gilz*, *Igfbp1*, *Fkbp51* and *Dusp1* compared with vehicle-treated group, while 5α-THB had a more limited effect in that it up-regulated the abundance of mRNAs of only *Fkbp51* and *Igfbp1* at 10 μM.

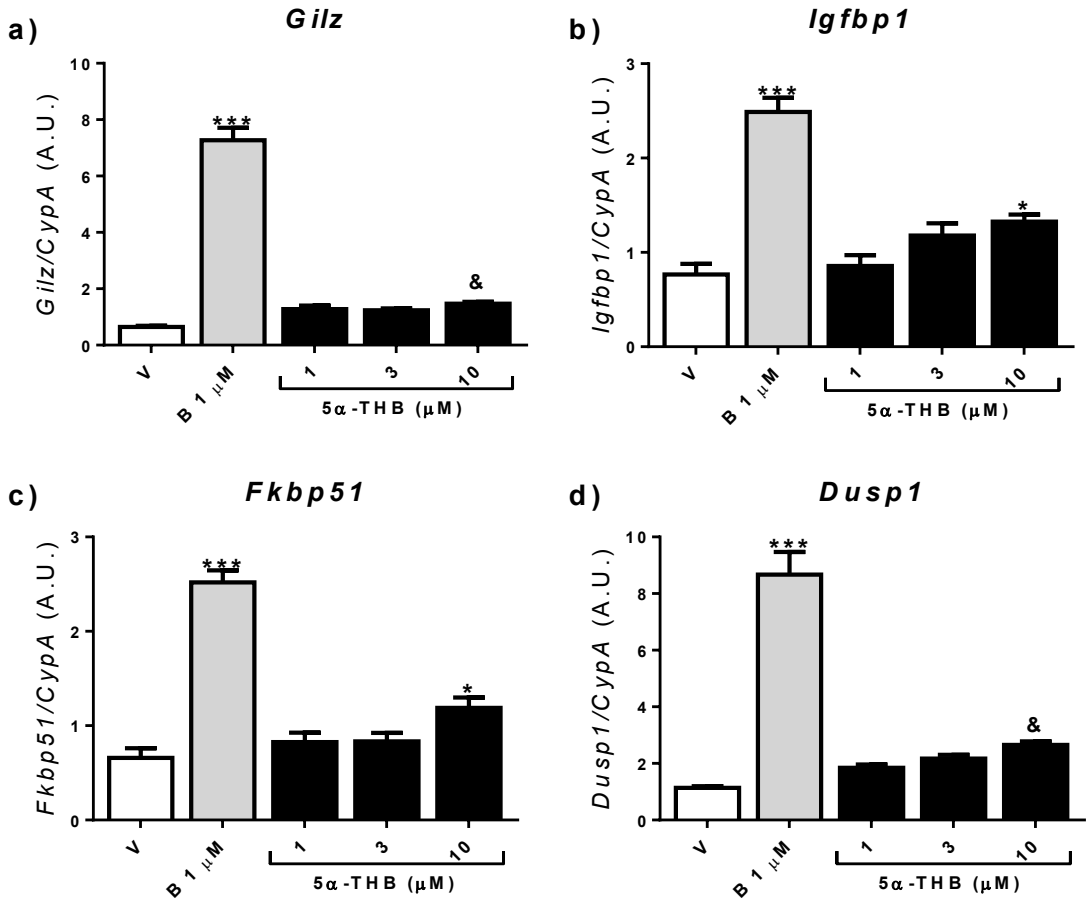

Figure 6.4 5α-Tetrahydrocorticosterone (5α-THB) and corticosterone (B) differentially affected the abundance of transcripts of selected genes in A549 cells. Quantification by real-time PCR of the abundance of transcripts of (a) *Gilz*, (b) *Igfbp1*, (c) *Fkbp51* and (d) *Dusp1* in response to incubation either with vehicle (V), B (1 μM) or increasing concentrations of 5α-THB (1-10 μM) for 2 hours. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; *** = $p < 0.0001$, * = $p < 0.05$, & = $0.1 < p < 0.05$ vs V; N = 6. A.U. = arbitrary unit.

6.3.3 Effects of 5α-THB on the activation of steroid-responsive reporters in A549 cells

6.3.3.1 Steroid responsiveness of reporter plasmids

As shown in Figure 6.5, B (1 μM) induced the activation of both reporters. The induction was greater when mGR was co-transfected with the plasmids. In the absence of mGR, the GR antagonist RU486 was able to significantly block the action of B at both concentrations studied for both reporters (a and b), however when mGR was co-transfected it could do so only when present at a concentration of 1 μM (c and d).

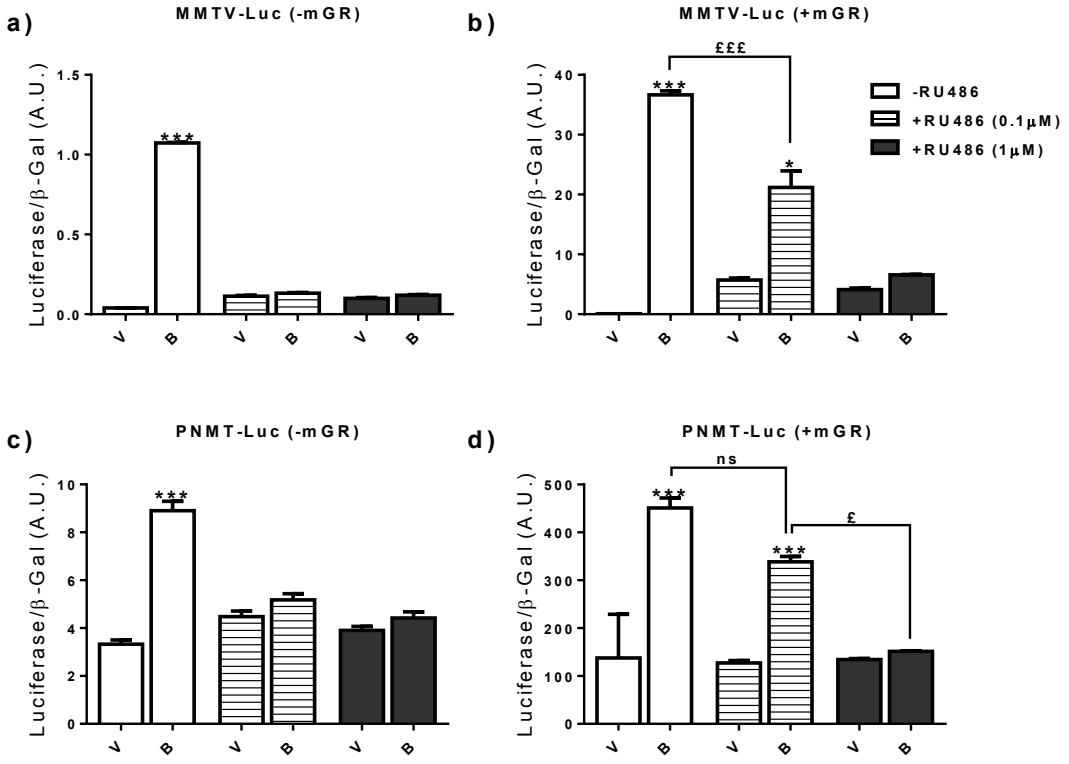

Figure 6.5 Corticosterone (B) activated the reporters MMTV-Luc and PNMT-Luc in a glucocorticoid receptor (GR)-dependent manner. Activation of the reporters (a) and (b) MMTV-Luc and (c) and (d) PNMT-Luc by B (1 μM), either in the absence (-mGR) or presence (+mGR) of co-transfected murine GR and with or without the GR antagonist RU486 as indicated. N = 3 analysed by one-way ANOVA with Tukey's post-hoc test. *** = $p < 0.0001$, * = $p < 0.05$ vs V; £££ = $p < 0.0001$, £ = $p < 0.05$, ns = not significant. Data are mean ± SEM. β-Gal = β-Galactosidase activity, A.U. = arbitrary unit.

6.3.3.2 Effect of 5α-THB on activation of reporter plasmids

MMTV-Luc

As shown in Figure 6.6, in the absence of co-transfected mGR, B increased the expression of the MMTV-Luc reporter, compared with cells treated only with vehicle (represented as 0 on the x axis), in a concentration-dependent manner at a concentration equal or greater than 0.1 μM; in the presence of mGR, B induced the expression of the reporter at any concentration tested (0.01-10 μM). 5α-THB increased the expression compared with the vehicle group only at 10 μM in absence of mGR, and at 3 and 10 μM in presence of co-transfected mGR.

PNMT-Luc

As shown in Figure 6.7, B activated the PNMT-Luc reporter when given at a concentration equal or greater than 1 μM in the absence of co-transfected mGR, compared with vehicle (represented as 0 on the x axis); in the presence of mGR, the minimal concentration that increased expression of PNMT-Luc was 0.01 μM. 5α-THB did not have any effect.

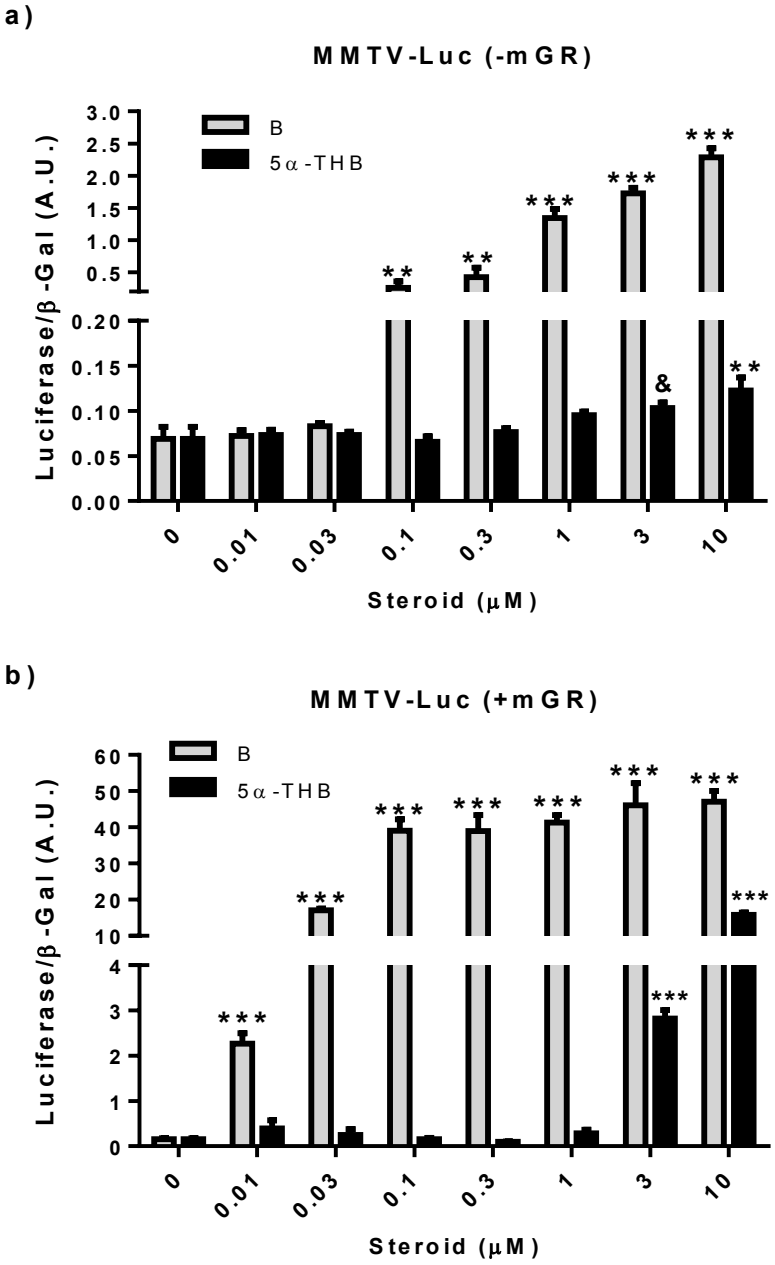

Figure 6.6 5α-Tetrahydrocorticosterone (5α-THB) only activated the MMTV-Luc reporter at 3 and 10 μM, whereas corticosterone (B) did so in a concentration-dependent manner. Quantification of luciferase activity (normalised to β-galactosidase (β-Gal) activity) in response to increasing concentration of steroids, either in the absence (a, -mGR) or presence (b, +mGR) of co-transfected murine GR (mGR). N = 6 analysed by one-way ANOVA with Tukey's post-hoc test; *** = $p < 0.0001$, ** = $p < 0.001$, & = $0.1 < p < 0.05$ vs V (0 in the x axis). Data are mean ± SEM. A.U. = arbitrary unit.

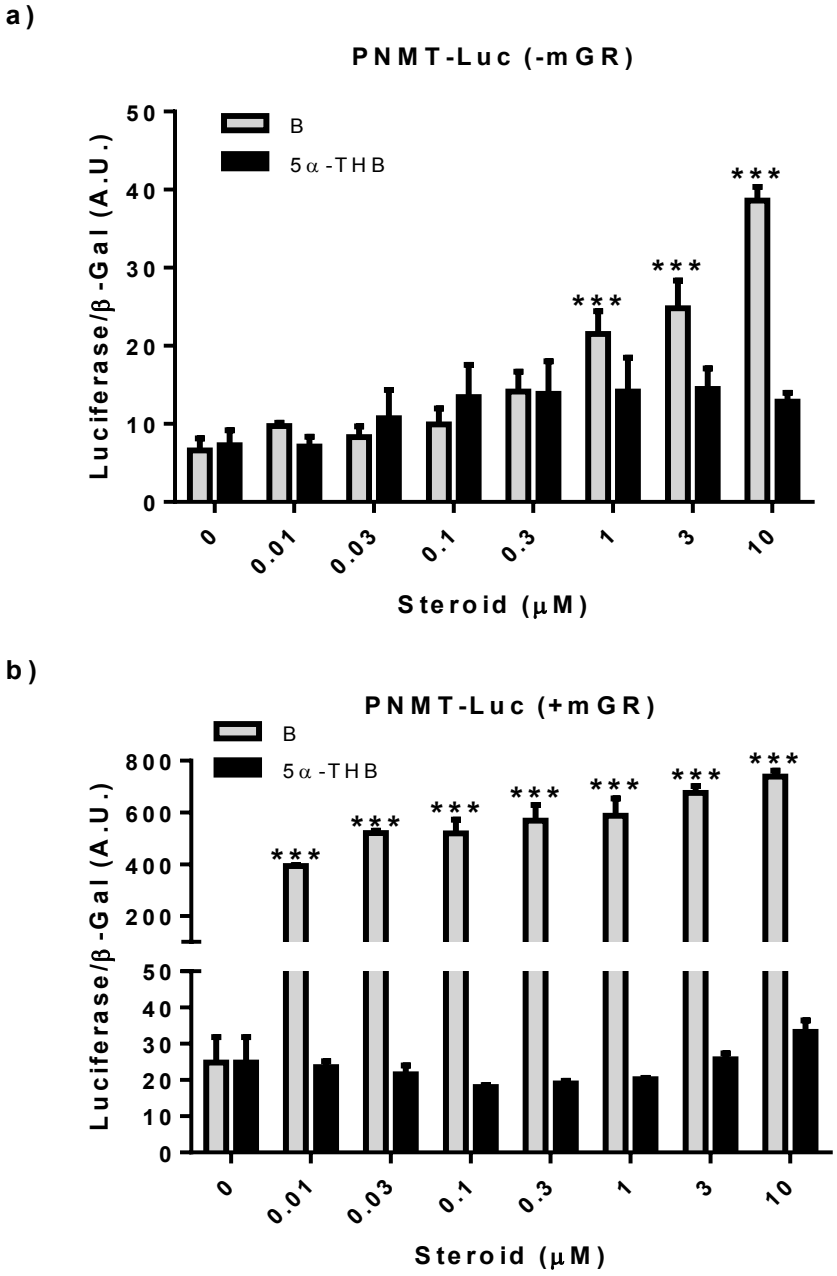

Figure 6.7 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not activate the PNMT-Luc reporter. Quantification of luciferase activity (normalised to β-Galactosidase (β-Gal) activity) in response to increasing concentration of steroids, either in the absence (a, -mGR) or presence (b, +mGR) of co-transfected murine GR (mGR). N = 6 analysed by one-way ANOVA with Tukey's post-hoc test; *** = $p < 0.0001$ vs V (0 in the x axis). Data are mean ± SEM. A.U. = arbitrary unit.

6.3.4 Effects of 5α-THB on abundance of transcripts of endogenous genes in BWTG3 cells

6.3.4.1 Evaluation of the model

The murine hepatoma cell line BWTG3 was responsive to dexamethasone (dex, 100 nM) and B (1 μM) in that they increased abundance of mRNAs of the *Tat* gene after 4 and 6 h of treatment compared with vehicle (V)-treated cells (Figure 6.8).

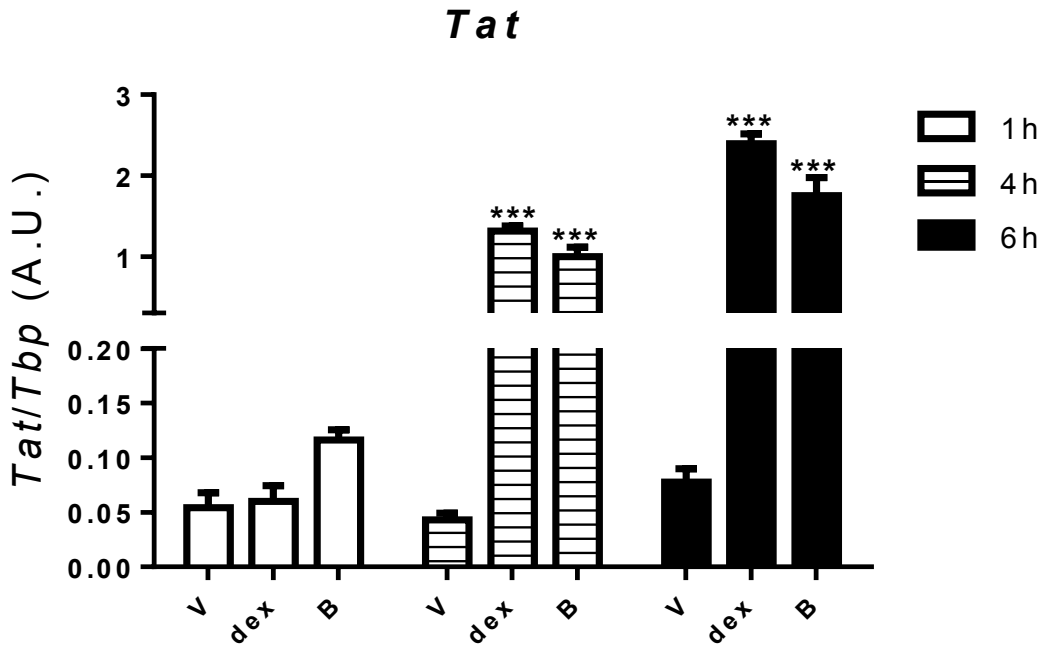

Figure 6.8 Dexamethasone (dex) and corticosterone (B) increased the abundance of transcripts of the gene encoding the metabolic enzyme tyrosine aminotransferase (*Tat*). Quantification by real-time PCR analysis of transcripts of *Tat* in BWTG3 cells treated either with vehicle (V) or steroids (dex, 100 nM; B, 1 μM) as indicated. N = 3 analysed by one-way ANOVA with Tukey's post-hoc test. *** = $p < 0.0001$ vs V. Data are mean ± SEM. *Tbp* = TATA-box binding protein; A.U. = arbitrary unit.

6.3.4.2 Effects of 5α-THB on abundance of transcripts of endogenous genes

6.3.4.2.1 Time-dependent effect of 5α-THB on *Tat*

In BWTG3 cells B increased abundance of mRNAs of *Tat* in a time-dependent manner compared with the vehicle (V) group, whereas 5α-THB did not (Figure 6.9)

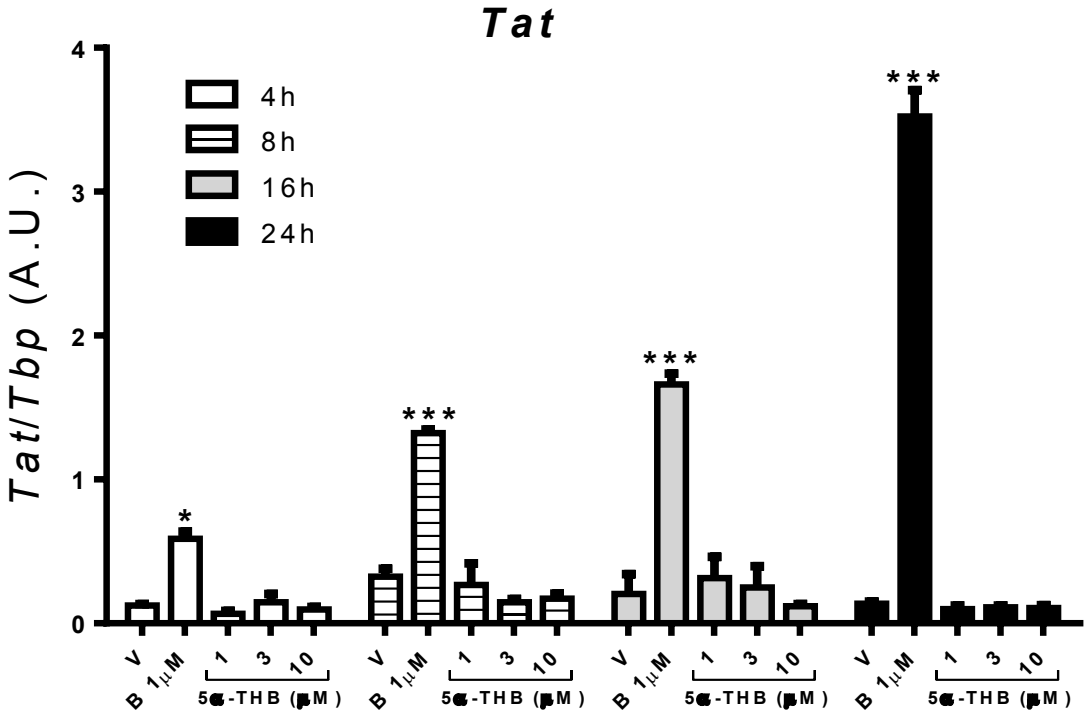

Figure 6.9 5α-Tetrahydrocorticosterone (5α-THB) did not affect the abundance of mRNAs of tyrosine aminotransferase (*Tat*), whereas corticosterone (B) increased it in a time-dependent manner. Quantification by real-time PCR analysis of transcripts of *Tat* in BWTG3 cells treated either with vehicle (V) or steroids as indicated for increasing period of time. N = 6 analysed by one-way ANOVA with Tukey's post-hoc test. *** = $p < 0.0001$, * = $p < 0.05$ vs V. Data are mean ± SEM. *Tbp* = TATA-box binding protein; A.U. = arbitrary unit.

6.3.4.2.2 Effects of 5α-THB on *Gilz*, *Igfbp1* and *Ggt1*

As shown in Figure 6.10, analysis of the effect of steroidal treatments on selected steroid-responsive genes revealed that B (1 μM) increased the abundance of transcripts of *Gilz*, *Igfbp1* and *Ggt1* compared with the vehicle-treated group, while 5α-THB did not.

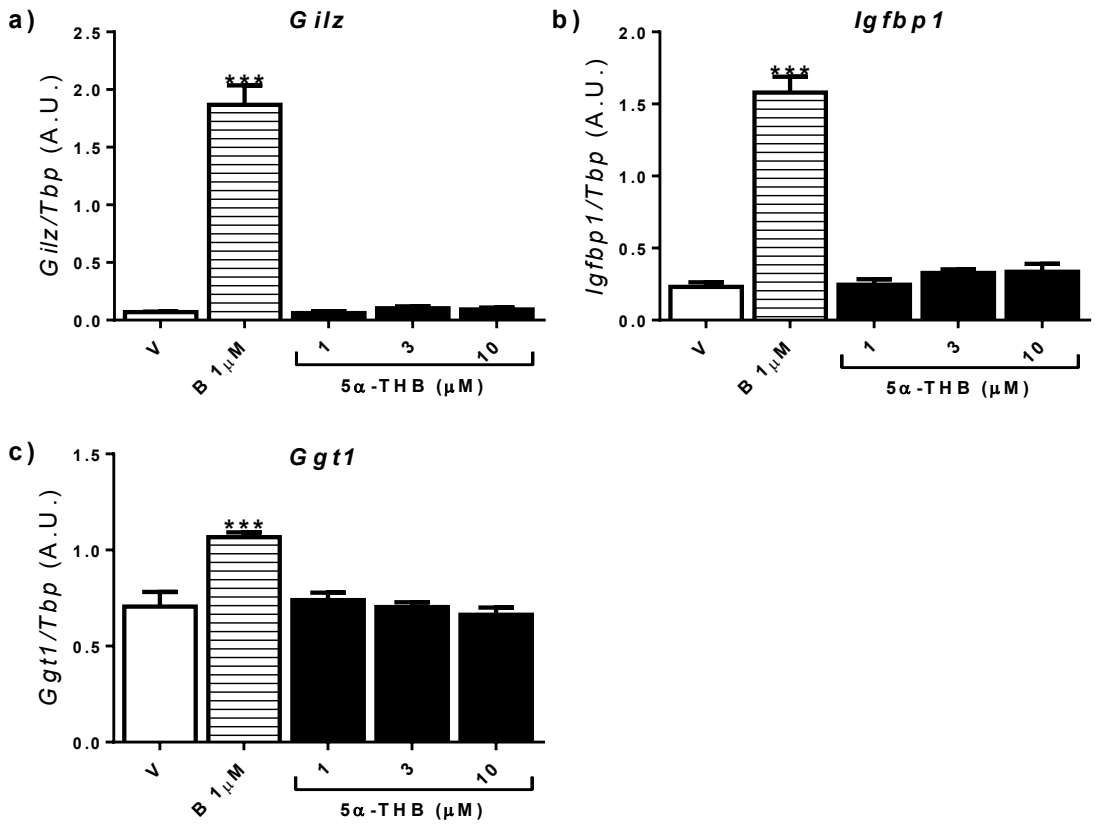

Figure 6.10 5α-Tetrahydrocorticosterone (5α-THB), unlike corticosterone (B), did not increase the abundance of transcripts of steroid-responsive genes in BTWG3 cells. Quantification by real-time PCR analysis of abundance of transcripts of (a) *Gilz*, (b) *Igfbp1* and (c) *Ggt1* in BWTG3 cells treated either with vehicle (V) or steroids for 16 hours as indicated. Data (mean ± SEM) were analysed with one-way ANOVA followed by Tukey's post-hoc test; *** = $p < 0.0001$ vs V; N = 6; Tbp = TATA-box binding protein, A.U. = arbitrary unit.

6.3.4.2.3 Effects of 5α-THB on the modulation of transcripts abundance by B

As shown in Figure 6.11, increasing concentrations of 5α-THB reduced the up-regulation induced by B (1 μM) of the abundance of transcripts of *Tat* in a concentration-dependent manner; the maximal effect was seen at the concentrations of 1, 3 and 10 μM, at which the 5α-reduced steroid decreased it by approximately 50% compared with cells treated only with B. An effect was also seen on the B-induced increase of the abundance of mRNAs of *Ggt1*, which was reduced by approximately 30% by the presence of 5α-THB at 3 μM.

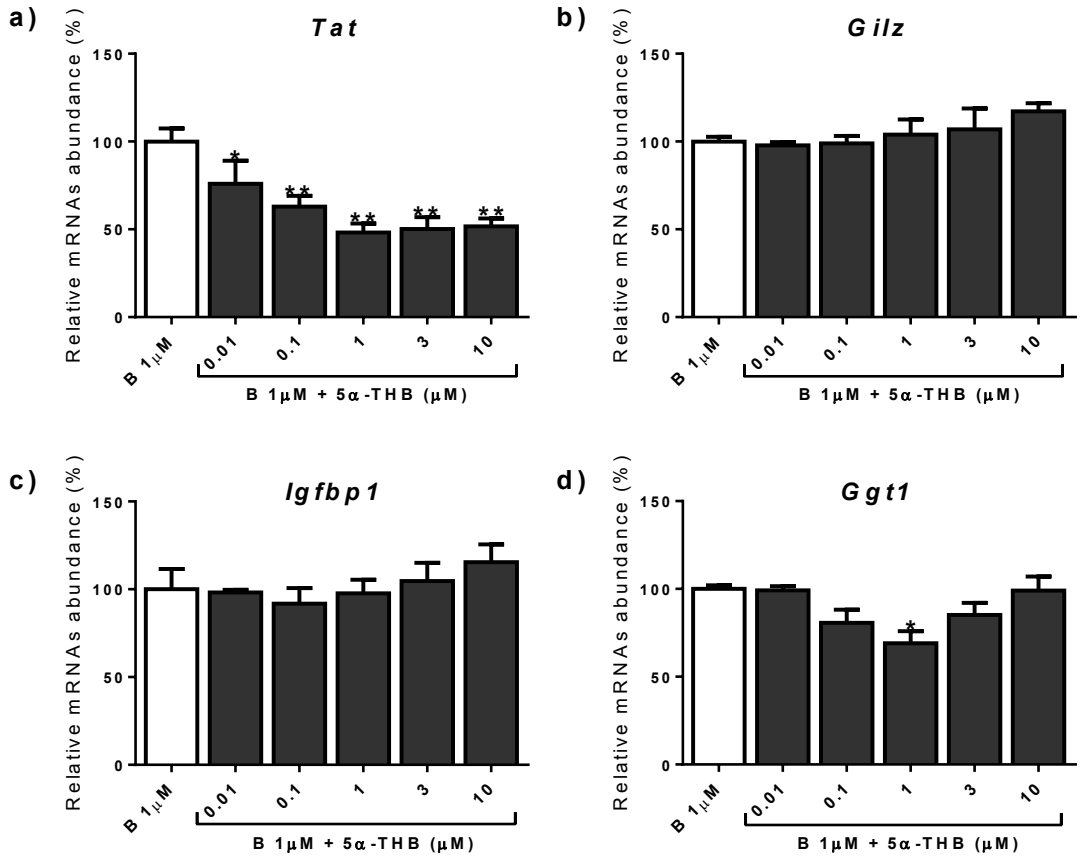

Figure 6.11 5α-Tetrahydrocorticosterone (5α-THB) affected the modulation of selected steroid-responsive genes by corticosterone (B) in BWTG3 cells. Quantification by real-time PCR of the effects of the presence of 5α-THB on the modulation of the abundance of transcripts of (a) *Tat*, (b) *Gilz*, (c) *Igfbp1* and (d) *Ggt1* by B after 16 hours of treatment. Data (mean ± SEM) were represented as relative transcription compared to B-treated group set as 100%, and were analysed by one-way ANOVA followed by Tukey's post-hoc test; ** = $p < 0.001$, * = $p < 0.05$ vs B 1 μM; N = 6.

6.4 Discussion

In the present chapter, the effects of 5α-THB on molecular signalling pathways involving GR were analysed, and it was shown that the 5α-reduced steroid is a weaker direct modulator of these pathways compared with B. Indeed, 5α-THB induced phosphorylation of GR, expression of steroid-responsive reporters, and affected the abundance of transcripts of endogenous genes in A549 cells only weakly. Furthermore, it did not affect the abundance of transcripts of selected genes in the hepatic cell line BWTG3, in contrast to B. However, when cells were treated with 5α-THB and B simultaneously, 5αTHB reduced the effects of B on the metabolic gene tyrosine aminotransferase (*Tat*), and to a lesser extent also of γ-glutamyltransferase 1 (*Ggt1*).

Previous chapters suggested that GR may not be the principal target of 5α-THB, as a consequence, in this one, a more direct approach to understanding if and how the compound affects GR has been employed. When applying this kind of strategy, common read-outs used in the literature are phosphorylation of GR and expression of endogenous or transfected genes known to contain glucocorticoid responsive elements (GREs) and respond to GCs; therefore, the present investigation aimed to analyse these processes.

GR undergoes a variety of post-translation modifications, which in turn modulate its activity. Phosphorylation was the first covalent modification identified, and at least six serine residues (Ser113, Ser141, Ser203, Ser211, Ser226 and Ser404) are subjected to phosphorylation on human GR, and these sites are conserved in rats and mice. GR displays a basal level of phosphorylation and becomes hyper-phosphorylated once bound to the ligand, with the extent of phosphorylation depending on the nature of the ligand (Avenant, Kotitschke et al. 2010; Avenant, Ronacher et al. 2010; Oakley and Cidlowski 2010). Phosphorylation of GR often changes its transcriptional activity in a gene-selective manner, and it is thought to influence the interaction with co-regulators (Chen, Dang et al. 2008). A study reported that the transcriptional activity of GR is correlated with a 10-fold increase in the phosphorylation state of Ser211 in the N-terminus of the receptor (Ismaili and

Garabedian 2004), while phosphorylation of Ser226 seems to induce cytoplasmic GR retention and increase its degradation (Wang, Frederick et al. 2002; Blind and Garabedian 2008). Since these links have been made, in the quest for alternative GCs, often the ability of candidates to induce phosphorylation of Ser211 has been investigated as read-out for their ability to promote activation of gene transcription, such as in the study of Compound A (CpdA). This molecule has been shown not to promote phosphorylation of Ser211, and this behaviour has been correlated with the absence of induction of promoters such as that present in MMTV-driven reporters (De Bosscher, Vanden Berghe et al. 2005). As a consequence, molecules inducing no phosphorylation or a differential pattern of post-translational modifications of GR may also be more likely to have a more selective and safer pharmacological profile. For this reason, the effects of 5α-THB on phosphorylation of Ser211 were investigated. Other phosphorylation sites were also analysed, namely Ser203 and Ser226; however, the antibodies used returned a high non-specific signal and, after extensive unfruitful trials aimed at improving the results, it was decided that the best approach was to focus only on Ser211.

Phosphorylation is a rapid process that has been shown to reach a peak at 1 h upon stimulation with steroids in COS-1 cells (Lynch, Rajendran et al. 2010). Consequently, the study presented in this chapter initially analysed this event at the same time point, confirming that indeed B strongly induced phosphorylation of Ser211. In contrast, treatment with an equivalent concentration of 5α-THB did not. This was not entirely unexpected. 5α-THB has been shown to possess anti-inflammatory properties and reduced metabolic side-effects in previous studies (Livingstone, Sykes et al. 2014), and the investigation on which this thesis is based has revealed that the compound affected *in vivo* and *in vitro* signalling pathways classically associated with steroids in a weaker, more restricted manner than B. This suggested also a potential weaker action on post-translational modifications of GR, since they have been linked to activation of downstream signalling pathways (Plumb, Robinson et al. 2013). From a technical point of view, there are a few experimental caveats to address. First, it would be of interest to investigate the nature of the double band obtained in the Western blot experiments. This is perhaps due to the presence of

multiple GR isoforms carrying other post-translational modifications, as shown also in another cell type (Lynch, Rajendran et al. 2010). Second, the quantification analysis performed on the data presented here decreased the reliability of the results, since calculating the ratio between ratios increases the variability of the data, requiring greater numbers of replicates. However, it has not been possible to quantify both GR and its phosphorylated form on the one gel.

Despite the above-mentioned findings, we were prompted to deepen the investigation into the phosphorylation of Ser211 because of a recent published study which showed that two potent non-steroidal ligands of GR caused delayed phosphorylation and induction of gene expression in comparison with classical agonists such as dexamethasone (Trebble, Woolven et al. 2013). As a consequence, a time-course study of Ser211 phosphorylation in response to increasing concentrations of 5α-THB was performed. While this analysis did not reveal a time-dependent phosphorylation pattern caused by the 5α-reduced steroid, it did show that the compound caused phosphorylation after 1 h treatment at 10 μM. B was confirmed to strongly induce phosphorylation, which was time-dependent. This result reinforced the idea that 5α-THB is a weaker modulator of GCs-responsive signalling pathways compared with classical steroids. In order to understand if the weak effect on phosphorylation had any functional consequences, the abundance of transcripts of steroid-responsive genes was analysed after 2 h of treatment. The products of the two genes *Gilz* and *Dusp1* are important proteins that mediate part of the anti-inflammatory effects of GCs (Clark 2003; Yang, Zhang et al. 2008; Ayroldi, Cannarile et al. 2012; Plumb, Robinson et al. 2013; Lim, Park et al. 2014). As expected, B strongly increased the amount of transcripts of these two genes. In accordance to a weak effect on phosphorylation, 5α-THB did not significantly increase the abundance of mRNAs of the genes, even though a trend was seen at the highest concentration (10 μM). Recently, work by a collaborator using microarray analysis showed increased expression of *Dusp1* in response to 5α-THB (1 μM) in unstimulated human blood-derived monocytes (Dr. Alistair Jubb, personal communication), suggesting that the behaviour of the compound may be cell-specific. However, it would also be of interest to study the effects of the two steroids on the expression of these genes upon

stimulation of the A549 cells with inflammatory stimuli, as it is plausible that the results will be different since both genes are actively involved in inflammation. A strong effect of B was also seen on the abundance of transcripts of another two classical targets for steroidal hormones, the genes *Fkbp51* and *Igfbp1*. Also in this case, 5α-THB had a weaker, albeit significant, effect at 10 μM. The protein FKBP51 is an immunophilin that binds to the GR complex in the absence of ligand by interacting directly with the molecule HSP90. Upon ligand binding, a correlated protein, FKBP52, switches position with FKBP51 promoting the activation of GR and its translocation to the nucleus (Guess, Agrawal et al. 2010). The expression of *Fkbp51* is known to be up-regulated by GCs and it is seen as a negative feedback for attenuating the responses induced by exposure to steroids (Plumb, Robinson et al. 2013). Therefore, 5α-THB may induce, to a small extent, this kind of feedback. Trans-activation by GR of the other gene investigated, *Igfbp1*, is known to be reliant on phosphorylation of Ser211 (Trebble, Woolven et al. 2013), and therefore it is not surprising that B increased the transcripts in a stronger manner than 5α-THB.

Modulation of GR phosphorylation by ligands is an important factor in determining the functional properties of the receptor, in that it alters the ability of the receptor to bind co-regulators, such as MED14, which in turn determine its effects on transcription. However, post-translational modifications are only one of the ways in which the function of GR is controlled. Indeed, the extent to which receptor ligands affect gene transcription depend also on the number of GREs found on the target genes, and on their sequences; and it is becoming more evident that the majority of them are not simple, canonical GREs as previously thought (So, Chaivorapol et al. 2007; Kaur, Chivers et al. 2008). This entails a further layer of complexity regarding how GR controls gene expression. The genes analysed in A549 cells have been shown to contain different types of GREs, with *Gilz*, for example, having four consensus GREs and *Igfbp1* harbouring instead non canonical GREs at which GR occupancy is lower compared with *Gilz* (Chen, Dang et al. 2008). In addition, also *Dusp1* does not appear to contain simple GREs (Kaur, Chivers et al. 2008). Interestingly, studies of the GREs contained in the *Igfbp1* gene have concluded that the strongest GRE for GR binding in response to dexamethasone is found in the first

intron rather than in the promoter region (Luo, Lu et al. 2013); on the one hand, the fact that 5α-THB increased the transcription of this particular gene, and not of others, may be due to a preference of the receptor through which it acts for this specific strong GRE, perhaps not present on other genes; on the other hand though, the fact that 5α-THB is less effective compared to B, may be due to receptor binding to the less strong GREs, or to a less effective binding to the strong one. Furthermore, the transcriptional up-regulation induced by GCs of *Gilz* and *Igfbp1* relies on a different type of co-regulator, making the latter, but not the former, dependent on the presence of MED14 (Chen, Dang et al. 2008). This complexity is further increased by the fact that the co-regulators vary also in a cell-dependent manner. Therefore, while it is reasonable to try to find a correlation between the degree of phosphorylation of Ser211 induced by 5α-THB and its transcriptional effects, other factors may be playing an important role in determining the behaviour of the compound, for example, the availability of co-regulators in A549 cells; the picture needs to be completed with further investigations.

In order to clarify if one of the determining factors of the behaviour of 5α-THB was the types of GREs in the promoter regions of target genes, the transcriptional responses to the compound of reporters containing different GREs were analysed. The cell line A549 was chosen as it expresses GR, however, because the effect induced by 5α-THB may have been weak, two approaches for investigating the dependency on GR were tested on the system: over-expression of GR and utilisation of the antagonist RU486. Both methods were efficacious in showing that the effects of B were GR-dependent, as expected. These results suggested that the model was suitable for the investigation of 5α-THB as a modulator of GR-activated signalling pathways, and provided two options for studying this relationship. While B strongly induced the expression of both reporters, 5α-THB selectively increased the expression of MMTV-Luc but not of PNMT-Luc reporter; furthermore, the induction was very weak in the absence of co-transfected hGR, and became stronger when the receptor was over-expressed, but in both cases was not comparable to that of B administered at the same concentrations. The findings point to a GR-dependent effect of 5α-THB, and the decision to test this possibility by over-expressing hGR instead

of using the antagonist RU486 was made because the effect in the absence of co-transfected hGR was of a very limited amplitude, making the over-expression approach more suitable. Had 5α-THB displayed a stronger effect, the experiments would have been repeated using the antagonist.

In line with a role of phosphorylation of Ser211 in promoting GR transcriptional activity, it has been shown that phosphorylation of Ser211 is necessary for maximal activation of the GR dimer-dependent synthetic promoter MMTV (Avenant, Kotitschke et al. 2010). It is therefore not surprising that the two steroids differed in their efficiency. In addition, while the promoter found on the MMTV-Luc reporter requires dimerisation of GR, the one found in PNMT-Luc reporter requires a more complex interaction between GR and other proteins (Adams, Meijer et al. 2003). Indeed, studies done employing a murine model harbouring a dimerisation-deficient GR (GR^{dim}) showed that the receptor is likely to induce the formation of so-called non-conventional dimers, or even of multimers, involving other transcription factors and/or hormone receptors. The MMTV-driven luciferase reporter contains a consensus palindromic GRE which is highly induced by the wild type GR, but not by GR^{dim}, while the PNMT-driven luciferase reporter achieves greater activation by the GR^{dim} than the wild type GR, suggesting alternate binding of GR to the promoter. It can be hypothesized that while B is able to induce conformational changes of GR in accordance with the formation of both classical dimeric structures and alternative complexes, and therefore induces both reporters, 5α-THB is only capable of inducing the formation of the former. The fact that this happened more strongly when GR was over-expressed indicates that the physiological level of GR found in A549 cells is not sufficient for gene induction by this compound. This may be due to the fact that 5α-THB is not as potent as B, and therefore the concentration used was too low for the drug to mediate strong effects. However, higher concentrations within the medium can induce toxicity and so maximal efficacy cannot be reliably tested. In corroboration, the response seen with the highest concentration was lower than with B.

The data presented in this chapter are somehow in contrast with some earlier studies in which 5α-THB was shown to induce MMTV-Luc reporter with a comparable potency to B (McInnes, Kenyon et al. 2004). The difference between the two investigations is the cell type used for the experiments. McInnes *et al.* worked with HeLa cells, while the results described in this chapter were obtained by using A549 cells. GR transcriptional activity is largely determined by the presence of co-factors, which are often expressed in a cell- and gene-specific manner (So, Chaivorapol et al. 2007; Zhang, Jonklaas et al. 2007). The cell system used in this chapter may have fewer of the important co-regulators required by 5α-THB to activate the MMTV promoter, or be devoid of them inducing the compound to use alternative ones not as efficiently. Nonetheless, the results presented here showed a clear difference between the behaviour of B and 5α-THB, with the latter seemingly lacking the ability to strongly induce GRE-driven expression.

In order to investigate what kind of transcriptional changes 5α-THB may promote in metabolic tissue, the changes in the abundance of transcripts of selected genes in response to the steroid were studied in a metabolic cell line. The mouse hepatic cell line BWTG3 was chosen in order to follow previous studies of the properties of 5α-THB in a mouse model (Yang, Nixon et al. 2011); in addition, this cell line expresses GR, and has been used for the investigation of the properties of another putative selective GR modulator, Cpd A (Robertson, Allie-Reid et al. 2009; Visser, Smith et al. 2010). The gene *Tat* was chosen as a model gene to analyse because it contains GREs, is induced by GCs, and, catalysing a crucial step in gluconeogenesis (Grange, Roux et al. 1991), is responsible for the onset of some of the side effects seen during treatment with anti-inflammatory steroids. Its transcription is promoted by the GR binding to the DNA as a homodimer, a mechanism that can be seen as a representation of the classical trans-activation way through which GR works. In the present chapter, the analysis of the abundance of transcripts of *Tat* after treatment with B and 5α-THB showed that the latter did not affect it, while it was confirmed that a classical GC (B) was able to significantly increase it and did so in a time-dependent manner. Investigation of other GC-responsive genes revealed a similar scenario in that treatment for 16 h with B up-regulated the amount of transcripts of

Gilz and *Igfbp1*, as shown in A549 cells, and *Ggt1*, while 5α-THB did not. These results suggest that 5α-THB does not promote binding of GR on endogenous "positive" GREs involved in potentially deleterious metabolic pathways, unlike B. While this scenario suggests a potential positive absence of stimulation of GRE-driven transcription by 5α-THB, one needs to remember that induction of transcription accounts also for some of the anti-inflammatory properties displayed by the conventional GCs. A compound that lacks this ability is also likely to have more limited anti-inflammatory effects.

This work is in accordance with the results obtained previously in mice by Yang et al. (Yang, Nixon et al. 2011), in which acute administration of 5α-THB did not increase *Tat* expression in liver, contrary to B. However, the results presented here are in contrast with those obtained by another study which showed that 5α-THB could significantly increase transcription of *Tat* in a rat liver cell line (H4IIE), after a 16-hour treatment (McInnes, Kenyon et al. 2004). As in the case of the discrepancies seen with the MMTV-Luc reporter, the explanation for these dissimilarities may lie in the different cell lines used. The co-regulators necessary for transcription of *Tat* in H4IIE cells may not be present in the BWTG3 cell line. The same phenomenon may account also for the contrasting results regarding the effects of 5α-THB on transcript abundance of *Igfbp1* in A549 and BWTG3 cells. However, it is worth noting that there is a certain degree of consistency between the two models regarding the promoters not targeted by 5α-THB; indeed, the lack of effects of the compound on PNMT-Luc activation in A549 cells, and on the abundance of mRNAs of *Tat* in BWTG3 cells, may be explainable by the fact that both promoters contain palindromic GREs, whilst, for instance, the MMTV-Luc reporter, which is activated by 5α-THB, contains GRE ½ sites. Therefore, 5α-THB could regulate preferentially genes harbouring these types of GREs which are considered non-canonical. In addition, and interestingly, a study showed that the efficiency with which different steroidal compounds trans-activate the same GC-responsive gene (in this case *Gilz*) is regulated by different parameters in different cell types. In effect, while in A549 cells the transcriptional activation was dependent on the extent of promoter occupancy by a given compound, in U2O cells the determinant factor for the same

compound was the extent to which nuclear localisation of GR was induced (Hadley, Louw et al. 2011). This is to remark on the fact that a compound is not only likely to behave differently from another that has intrinsic divergent properties, but also from itself when studied in different cellular environments. It is therefore not surprising that 5α-THB did not show exactly the same behaviour in different cell types, also considering that being a weak agonist regarding certain responses, it may be more susceptible to the changes in different factors. Nonetheless, the fact that the results presented in this chapter are in accordance with results obtained *in vivo*, indicates that they are likely to be relevant.

In pharmacology, a partial agonist is defined as a compound that binds and activates a given receptor inducing a response which is less efficacious than that of a full agonist acting on the same receptor. When partial and full agonists are present together, the former may behave like a competitive antagonist, competing for the occupancy of the receptor and reducing its activation by the full agonist (Zhu 2005). 5α-THB has shown properties of a weak agonist throughout the experiments presented in this thesis, and with this in mind, it was decided to co-incubate B and 5α-THB with BWTG3 cells in order to analyse the possibility that the latter may behave like a competitive antagonist, and therefore show some of the characteristics of a partial agonist. Interestingly, 5α-THB did indeed antagonise the up-regulation of transcript abundance of *Tat* induced by the full agonist B, reducing it by approximately 50%. To a lesser extent, the phenomenon was also seen for the gene *Ggt1*. This result may provide a mechanism explaining the phenotype seen in mice harbouring a genetic disruption for the 5α-THB-producing enzyme 5α-reductase type 1 (5α-R1), which displayed a worse hepatic metabolic profile than wild type mice (Livingstone, Barat et al. 2014). Indeed, it may be possible that physiological concentration of 5α-THB, mainly produced in the liver, are able to decrease the metabolic effects of B as suggested form the experiments in BWTG3 cells. It would be of interest to perform a similar experiment analysing the effect of co-incubation of B and its metabolite on the reporters previously mentioned; this would provide information on whether the phenomenon is a broad one.

Exactly through which mechanism 5α-THB may accomplish the above-mentioned effects is an open question. The compound may affect any of the steps and processes involved in the cellular response to B, from ligand binding to nuclear translocation, to the binding to GREs. Indeed, 5α-THB may act directly by binding to GR and, as a consequence, reduce the amount of GR available to bind B; otherwise, 5α-THB may also reduce B-induced translocation of GR to the nucleus as seen with the antagonist ORG 34517 (Peeters, Ruigt et al. 2008). In addition, 5α-THB may work at the level of the DNA impeding the binding of GR to GREs, or disrupting fundamental changes required for initiation of transcription. Recently, it has been reported that proteins belonging to the negative elongation factor (NELF) complex are capable of reducing the trans-activation induced by GR of GRE-containing genes, such as *Gilz* and *Igfbp1*; this is achieved by binding of NELFs to the LBD of the activated receptor, therefore reducing its recruitment to GREs (Luo, Lu et al. 2013). A fascinating speculation would be that 5α-THB may recruit some of these factors to reduce the amount of GR bound to B reaching target genes. The fact that these molecules also differ in their inhibitory potential according to the type of GREs, and the type of cells, is a fact that make them even more interesting from the perspective of this study. A further possibility is that the 5α-reduced steroid is acting through a different isoform of GR, for example GR_{β}, which is known to have inhibitory effects on GC-induced gene transcription (Zhang, Clark et al. 2008; Guess, Agrawal et al. 2010). Regardless of the reasons behind the antagonistic behaviour of 5α-THB, the experiments in BWTG3 cells revealed both an intriguing and promising mode of action of the compound, which, if confirmed relevant in other models, may be exploited for the reduction of the metabolic side effects induced by classical steroids.

Some of these mechanisms, along with the results presented in Chapter 3 and 4, suggest the possibility that 5α-THB may act through a receptor which is not GR. Liver X receptors (LXRs) are important receptors controlling metabolism, and have been shown to bind to GREs and, when activated or over-expressed, to repress the activity of GR on metabolic genes (Demerjian, Choi et al. 2009; Nader, Ng et al. 2012); furthermore, another receptor important in regulating hepatic functions, pregnane X receptor (PXR), has been shown to bind to steroids (Banerjee, Robbins et

al. 2013; Gerbal-Chaloin, Iankova et al. 2013). This evidence makes the two receptors reasonable candidates for 5α-THB. Another putative receptor is the progesterone receptor (PR); PR has high homology with GR, in that the two are 90% identical in their DBD and 55% in the LBD, and despite being bound to different hormones, they can bind to and activate transcription from the same hormone responsive elements (HREs). One of the factors that determines the efficiency with which PR would induce a gene versus GR is the presence of certain co-regulators (Szapary, Song et al. 2008); it is possible that 5α-THB may activate transcription through PR, and do so in a less efficient manner than B because the genes studied here may require the presence of regulators which do not efficiently interact with PR. A broader investigation of gene expression in response to 5α-THB may be helpful to gain a clearer and fuller picture.

In summary, in this chapter the effects of 5α-THB on signalling pathways controlled by GR were investigated. The compound appeared to be a weak agonist, and despite the results obtained with the MMTV-Luc reporter suggesting an action through GR, the involvement of other receptors is not to be excluded. Repeating some of the experiments presented here by using siRNA technology against GR, or by employing the antagonist RU486, may clarify at least in part this aspect. Furthermore, studies that investigate other post-translational modifications of GR in response to 5α-THB treatment may be useful; in effect, some of them are important in determining the receptor transcriptional activity because they work in concert with the most commonly studied Ser211 (Avenant, Ronacher et al. 2010; Oakley and Cidlowski 2010). If 5α-THB does indeed bind to GR to deliver its effects, a lower affinity for the receptor in comparison with B may explain is weaker agonistic properties. This may be better clarified with molecular modelling or crystallography analyses aimed at studying the possible characteristics of an interaction between the compound and GR; this would also shed light on which co-regulators may or may not bind to the complex, increasing the understanding of which genes and cells may be targeted by 5α-THB.

Chapter 7

SUMMARY AND FUTURE WORK

Chapter 7: Summary and future work

The research presented in this thesis was born out of the necessity to deepen the understanding of the molecular mechanisms whereby 5α-THB works to reduce inflammation, and to investigate how molecular pathways associated with beneficial outcomes and deleterious side effects are affected by the compound. Since previous *in vivo* and *in vitro* published and unpublished studies showed that this compound is a more selective anti-inflammatory agent than both B, from which it derives, and the clinically used glucocorticoid hydrocortisone (HC) (Yang, Nixon et al. 2011; Livingstone, Sykes et al. 2014), the hypotheses being tested were that 5α-THB preferentially modulates inflammatory signalling pathways, and that it does so through GR.

7.1 5α-THB is an effective anti-inflammatory compound that may not work through GR

First of all, the work of this thesis confirmed previous *in vivo* and *in vitro* findings in that it showed that 5α-THB decreases inflammatory swelling associated with irritant dermatitis (Livingstone, Sykes et al. 2014), and suppresses the release of pro-inflammatory cytokines in primary cultures of murine bone marrow-derived macrophages (BMDMs) (Yang, Nixon et al. 2011), but in a weaker manner than B.

Most importantly, original contributions towards the understanding of the molecular mechanisms whereby 5α-THB works to resolve inflammation *in vivo* have been provided here. Indeed, for the first time, it has been shown that the compound acts more slowly than B, being effective after 24 but not 6 h of treatment in a model of irritant dermatitis. While the work focussed on analysing how 5α-THB mechanistically reduced inflammation at 24 h, some preliminary studies at 6 h showed that the compound is unable to decrease the infiltration of inflammatory cells. The reasons behind this phenomenon were not investigated, but they may relate to less efficient modulation, compared with B, of signalling pathways associated with the early phases of the response to the insult (croton oil in this case); for instance,

5α-THB may not be able to prevent the release of inflammatory mediators from the cells (such as endothelial cells, keratinocytes and fibroblasts) responsible for the initiation of the host response. Alternatively, the compound may be unable to prevent the activation of the microvasculature (that leads to vasodilation and increased permeability) that plays a pivotal role in the onset of swelling and infiltration of cells (Lee, Stieger et al. 2013). This and other processes may be instead controlled efficiently and rapidly by B; as an example, in Chapter 5 it is demonstrated that B, but not 5α-THB, reduced the abundance of transcripts of *eNos*, a gene encoding an enzyme that promotes vasodilation through the production of NO during both inflammation and angiogenesis. Another important mediator common to the two processes is VEGFα, which in the case of skin inflammation is released from damaged keratinocytes, and is a strong inducer of vascular remodelling (Huggenberger and Detmar 2011); *Vegfα* transcripts were decreased in inflamed ears by both compounds after treatment lasting 24 h; it would be of interest to analyse the same gene at the earlier time point to understand whether it is affected by B but not by 5α-THB. Investigation of the effects of 5α-THB on specific cell types present in the skin, such as keratinocytes and fibroblasts, would also help.

When the model was analysed for the molecular changes associated with the anti-inflammatory effects of 5α-THB at 24 h, the picture that emerged was one in which B and its 5α-reduced metabolite influenced similar pathways, in a similar way. The data suggested that the two compounds act mainly by down-regulating the abundance of transcripts of genes involved in the modulation of vascular permeability. Nonetheless, three major differences between the two were revealed:

- 5α-THB was more effective than B in reducing the infiltration of neutrophils
- 5α-THB increased the abundance of transcripts of *Dusp1* in inflamed ears, but B did not
- The GR antagonist RU486 prevented the effect of B on swelling but not that of 5α-THB

Whether these differences are sufficient for explaining the behaviour of 5α-THB is not yet known, but they pave the way for planning further research targeted to answer specific questions, related also to other models employed in this thesis.

Question 1: Which cell population represents the principal target of 5α-THB, and how is it affected by the compound?

Part of this question may be answered by an extended analysis (e.g. through FACS) of how the populations of inflammatory cells infiltrating the auricular tissue change in response to 5α-THB, in comparison with B, after administration for 6 and 24 h. While in the *in vivo* model of inflammation the effects on macrophages were not investigated, the data obtained with primary culture of murine BMDMs and the *in vivo* model of angiogenesis showed that 5α-THB also affects these cells. This population plays a vital role in inflammation, by releasing cytokines and chemokines; furthermore, it has been shown that, in *in vivo* models of psoriasis, macrophages are required for neutrophil recruitment (Stratis, Pasparakis et al. 2006; Wang, Peters et al. 2006). Despite the fact that irritant dermatitis and psoriasis are different dermatological conditions, macrophages are also recruited to the tissue after contact with an irritant, and play a pivotal role during the acute and reparative phases of the inflammatory response (Tang, Schlapbach et al. 2010); it is therefore a possibility that 5α-THB may be affecting neutrophil infiltration in part by modulating macrophage activity. Regarding the mechanisms whereby the compound may affect this cell population and others, this is still an open question. It is known that classical steroids work by influencing a broad range of cellular mechanisms, either genomic or non-genomic, and the overall picture emerging from the data of this thesis is one in which 5α-THB affects fewer of those tested than B. In Chapter 4, some preliminary data on how 5α-THB may interfere with one of the classical pathways with a central role in inflammation, NF-κB, have been obtained through Western blot analysis, and, in contrast to B, they show only a marginal effect of the compound. One possibility is that 5α-THB may be interfering in a stronger manner with the transcription factor AP-1 rather than NF-κB. A study suggested that the GR-mediated repression of the two factors is dependent on different domains of the

receptor (Bladh, Liden et al. 2005); it is therefore possible that 5α-THB may preferentially induce a conformation of GR, or another receptor, more suitable for the inhibition of AP1 than NF-κB. As an example, a new selective glucocorticoid receptor modulator, Compound A, has been shown to preferentially repress NF-κB-driven transcription rather than AP-1, showing that new selective compounds may not only be able to discern between GR-dependent gene activation and repression, but also between different types of repressive mechanisms (De Bosscher, Beck et al. 2013). Studies analysing the differential ability of 5α-THB to suppress transcription of AP-1-dependent versus NF-κB-dependent genes may be useful to clarify this point. Some preliminary experiments regarding this aspect have been previously conducted by my supervisors' group, and they provided surprising results; indeed, they showed that in human embryonic kidney (HEK) cells 5α-THB potentiated, in a GR-independent manner, the NF-κB- and AP-1-mediated expression of the luciferase gene induced by phorbol ester (Nixon 2011). While the lack of a dependency on GR is in accordance with the results presented in Chapter 3 and Chapter 4 of this thesis, more extensive investigations (analysing different cell types and inflammatory stimuli) are required to shed light on the potential mechanisms involving NF-κB and AP-1, and explain the anti-inflammatory properties of 5α-THB. Furthermore, GCs have been shown to suppress the production of pro-inflammatory cytokines at a post-transcriptional level (Clark, Dean et al. 2003), adding another putative mechanism whereby 5α-THB may work. In effect, GCs have the ability to induce the expression of tristetraproline (TTP), which binds to mRNAs of pro-inflammatory genes destabilising them (Kadmiel and Cidlowski 2013). The production of some cytokines has been shown to be dependent on post-transcriptional mechanisms, adding a layer of complexity that compounds controlling the inflammatory response may target (Zuckerman, Evans et al. 1991).

Question 2: To what extent are the anti-inflammatory properties of 5α-THB dependent on DUSP1?

GCs are known to suppress pro-inflammatory pathways, activated for instance by LPS, by stimulating the expression of DUSP1, which in turn inhibits p38 MAPK and

therefore the activation of AP-1 (Bhattacharyya, Brown et al. 2007). In addition, and as mentioned before, new selective regulators of GR have been found to act in this way (Joanny, Ding et al. 2012). In accordance with the findings presented in Chapter 3, whereby 5α-THB increased the abundance of transcripts of *Dusp1* in inflamed ears, previous results from my supervisors' group also showed increased abundance of DUSP1 protein in LPS-stimulated RAW264.7 cells after treatment with 5α-THB (Nixon 2011), and another preliminary study employing human blood-derived monocytes found that the compound did increase the abundance of transcripts of this gene (Dr. Alisdair Jubb, personal communication). Despite the fact that a broader investigation of the many signalling pathways involved in inflammation is necessary to gain a more complete picture of the differences between 5α-THB and classical steroids such as B, the employment of *Dusp1* -/- mice would be a good starting point. This would also allow the analysis of specific cell populations (e.g. BMDMs) isolated from these mice in order to determine the cellular target of the compound (for instance macrophages vs neutrophils).

Question3: Are the anti-inflammatory properties of 5α-THB independent of GR?

In Chapter 3, it was shown that the attenuation of ear swelling upon application of 5α-THB was not affected by the GR antagonist RU486. In addition, in Chapter 4 the same antagonist was shown not to prevent the reduction in the release of IL6 from BMDMs induced by 5α-THB. These results must be confirmed by using an alternative method to the pharmacological antagonism with RU486, both *in vivo* and *in vitro*. Mice in which GR is knocked down would be a useful tool, although it needs to be taken into consideration that only heterozygous mice can be used, as the total knock out of GR is lethal with mice dying hours after birth because of respiratory failure (Cole, Blendy et al. 1995). This would mean employing a model in which GR is still expressed, albeit to a lesser extent than in wild type animals. Another possibility would be to generate a conditional knock out mouse model where GR expression could be switched off whenever deemed appropriate. If contrary to what is suggested in this thesis, the effects of 5α-THB are found to be dependent on GR, targeted disruption of the receptor in difference cell populations of

the skin or the immune system, such as keratinocytes and macrophages/neutrophils, may be the strategy to employ. Previous *in vitro* studies showed that 5α-THB did bind GR (McInnes, Kenyon et al. 2004), and translocate it to the nucleus (Yang 2009). Interestingly, the translocation was slower than that induced by B, and after 24 h did not reach the same level. This could explain why 5α-THB acted more slowly, and was less potent than B, in the dermatitis model. If GR is indeed involved in the responses induced by 5α-THB, the lack of antagonism by RU486 in the dermatitis model would need to be explained. One possibility could be that the 5α-reduced steroid binds to a different site compared with RU486, or that perhaps its displacement is not possible because of strong interactions with the receptor. The former would perhaps also explain the additive effect of RU486 and 5α-THB on the release of IL6 from BMDMs. Molecular modelling and crystallography analysis would be useful tools that would provide potential explanations. However, a dependency on GR, most of all *in vivo*, must be first robustly proved, and if instead the anti-inflammatory effects are found not to be consistently dependent on GR, the investigation needs to move into the search for alternative receptors. Some possible targets are outlined below.

In addition to GR, other nuclear receptors have been shown to play a major role in skin homeostasis. Among them are the liver X receptor (LXR) and the pregnane X receptor (PXR) (Schmuth, Moosbrugger-Martinz et al. 2014). Both receptors have a role in controlling skin inflammation and keratinocyte function. LXR modulates numerous metabolic pathways, and some of its ligands are products of the oxidation of cholesterol, namely oxysterols, and other lipids (Nader, Ng et al. 2012). The isoform LXR beta is expressed in mouse epidermis, white blood cells and neutrophils, making it relevant to skin inflammation (Komuves, Schmuth et al. 2002; Chang, Shen et al. 2008; Man, Barish et al. 2008). Agonists of LXR have been shown to lower inflammatory cytokines and metalloproteinases (Mohan and Heyman 2003; Schmuth, Moosbrugger-Martinz et al. 2014), while LXR-β null mice show thinner epidermis than control animals due to decreased proliferation of keratinocytes (Chang, Shen et al. 2008). Topical activation of the receptor suppressed irritant and allergic contact dermatitis by lowering the production of

TNFα and IL1 (Schmuth, Moosbrugger-Martinz et al. 2014). Interestingly, application of LXR ligands with GCs prevented side effects induced by GCs such as atrophy of skin (Demerjian, Choi et al. 2009). Furthermore, activation/over-expression of LXR repressed the transcriptional activity of GR on metabolic genes, and it was found that endogenous LXR bound GRE (Nader, Ng et al. 2012). All this suggests a crosstalk between the GR and LXR, and it is tempting to speculate that the latter may be responsible for the anti-inflammatory effects of 5α-THB. It would be interesting to investigate what happens when 5α-THB is co-applied with other steroids known to cause dermal side effects such as skin thinning. Previous studies (Livingstone, Barat et al. 2014) showed that mice with a genetic disruption of 5α-R1 have a worse hepatic metabolic phenotype than wild type littermates. This may indicate a physiological role of 5α-THB in repressing the action of endogenous GCs, a role that may be played out through LXR, which is highly abundant in the liver.

Another interesting and important nuclear receptor found in the skin and immune cells is PXR, which regulates the metabolism of endogenous hormones and exogenous xenobiotic compounds by modulating cytochrome P450 enzyme expression. Together with the constitutive androstane receptor (CAR), PXR belongs to the group of xenobiotic receptors. These receptors have promiscuous ligand-binding properties (Banerjee, Robbins et al. 2013) and bind structurally different molecules. Indeed, they are activated by a broad range of compounds, among which are steroids, bile acids, dietary lipids, drugs such as antibiotics, progesterone and RU486. PXR is involved in the control of steroid hormone pathways as it is activated by C21 steroids. Interestingly, the phorbol ester phorbol-12-myristate-13-acetate (PMA), in combination with other inflammatory stimuli, has been found to greatly activate PXR (Gerbal-Chaloin, Iankova et al. 2013), and this is one of the compounds found in croton oil. PXR may have a role in KC proliferation in that it may suppress NF-κB, over-expression of which has been shown to decrease KC proliferation. Furthermore, repression of the NF-κB pathway suggests an anti-inflammatory role for this receptor (Gerbal-Chaloin, Iankova et al. 2013). Despite the fact that much still needs to be investigated about the role of PXR in skin

homeostasis and inflammation, data available so far point to it as an interesting drug candidate and as a potential receptor for 5α-THB.

More work is clearly needed to gain a clearer understanding of how 5α-THB works to resolve inflammation, and the availability of mice lacking the expression of receptors such as LXR and PXR, and of inhibitors of these two potential targets, offers good tools to expand our knowledge, either in *in vivo* or *in vitro* models. Furthermore, another important issue to address is about how far 5α-THB penetrates into the dermal layers. This would inform about potential systemic effects in cases of long-term topical administration. In order to address this, a plan is already in place to carry out mass spectrometry tissue imaging experiments in pig models, using a protocol developed in my supervisors' laboratory (Cobice, Mackay et al. 2013).

7.2 5α-THB possesses weaker anti-angiogenic and trans-activation properties in comparison with B

In an effort to try to understand whether 5α-THB inhibits angiogenesis, and activates molecular signalling pathways associated with side effects induced by GCs, an *in vivo* model of angiogenesis and *in vitro* cell systems were interrogated.

The sponge model of angiogenesis revealed that 5α-THB, at equipotent doses to B for anti-inflammatory properties (reduction of macrophage infiltration), inhibits angiogenesis in a weaker manner than B. The overall picture that emerged is one in which B and 5α-THB affect a smaller number of common molecular pathways than in the dermatitis model. For this reason, I would like to draw attention to those aspects of this model not significantly affected by the 5α-reduced steroid, and instead strongly perturbed by B.

5α-THB, unlike B, did not:

- reduce the number of vessels expressing αSMA (mature vessels)
- affect the abundance of transcripts of the majority of genes analysed, including genes involved in angiogenesis and the inflammatory response
- reduce the amount of collagen in sponges

The fact that 5α-THB had more limited effects than B suggests important functional differences. First of all, and as mentioned in Chapter 5, the lack of strong effects on the expression of αSMA, a marker of late-stage angiogenesis, indicates that 5α-THB may specifically not interfere with processes (e.g. migration of pericytes and pruning of the newly formed vascular network) that are characteristic of the final phases of new vessel formation. In contrast, the strong effects of B both on CD31 and αSMA expression point to a broader action of the compound. This suggests that B may be targeting more than one cell type, while 5α-THB may be more selective and acting mostly on endothelial cells, which have an active and central role in the early phases of angiogenesis. These results are promising, in terms of developing a safer therapy. Indeed, inhibition of the angiogenic process may represent a negative outcome when steroids are used to treat inflammatory conditions, and in particular topical ones such as dermatitis. A compound like 5α-THB that modulates fewer signalling pathways, and reduces only weakly the number of functional vessels, may have a lesser impact on the functionality of the tissue treated. This may be even more likely considering the dose of the 5α-THB necessary to inhibit angiogenesis was much higher than the dose displaying topical anti-inflammatory properties in the dermatitis model. In effect, this would suggest that the two effects may be dissociated.

From the aforementioned results, a question, that ought to be answered in future research, arises: which cells are targeted by 5α-THB during angiogenesis?
It is known that glucocorticoids suppress endothelial (Longenecker, Kilty et al. 1984; Sakamoto, Tanaka et al. 1987) and smooth muscle cell proliferation through mechanisms that are not completely clear, and may be both direct and indirect (Radke, Weber et al. 2004). In addition, dexamethasone has been shown to induce apoptosis of rat microvascular pericytes *in vitro* (Katychev, Wang et al. 2003). In the past decade the importance of these cells in angiogenesis has become increasingly evident (Bergers and Song 2005); for example, they can release VEGF. Studies suggest that apoptosis of pericytes may be linked with vascular regression (Katychev, Wang et al. 2003), and GCs have been known to induce the latter since the first experiments performed by Folkman *et al.* (Folkman, Langer et al. 1983). Although proliferation or apoptosis were not analysed in the work presented in this

thesis, we could hypothesize that one of the mechanisms by which B, but not 5α-THB, decreased angiogenesis, and in particular the expression of αSMA, may be through induction of apoptosis of pericytes which express this protein. The effect of B may have been also indirect though, through modulation of the inflammatory response. In effect, inflammation has been shown to stimulate expression of αSMA in pericytes (Nehls and Drenckhahn 1993; Armulik, Genove et al. 2011).

Angiogenesis and inflammation are two processes that share many mediators. As an example, molecules such as IL6, TNFα, VEGF and NF-κB promote angiogenesis (Conway, Collen et al. 2001), are released during inflammation, and are also suppressed by GCs (Barnes 1998; Wallerath, Witte et al. 1999; Beer, Fassler et al. 2000; Hasan, Tan et al. 2000; Grose, Werner et al. 2002; Blomme, Chinn et al. 2003). As a consequence, the inhibition of angiogenesis seen with B and 5α-THB may be due, in part, to their anti-inflammatory properties. However, the mechanisms through which they deliver this effect may be be very different, since B down-regulated the abundance of transcripts of pro-inflammatory genes while 5α-THB did not.

While one of the cellular targets of 5α-THB may be the endothelial cell population, it may also affect angiogenesis by modulating cells of the immune system. Indeed, 5α-THB and B decreased the proportion of macrophages in sponges to a similar extent, and this effect may provide a potential mechanism explaining the reduction in the number of vessels as macrophages have also pro-angiogenic properties. Whilst until a few years ago these cells were considered a homogeneous population secreting pro-inflammatory mediators and displaying phagocytic behaviour, compelling evidence has now emerged for the existence of at least two types of macrophages (Nahrendorf, Swirski et al. 2007; Bartneck, Peters et al. 2014). These are classified as either classically- or non-classically activated macrophages. The first group includes a population of cells that are pro-inflammatory, and that are activated by bacterial toxins such as LPS or stimuli such as INFγ produced by other leukocytes; the second population, by contrast, is anti-inflammatory and is able to

secret pro-angiogenic molecules such as IL4, TGFβ, IL8 and TNFα (Loke, Nair et al. 2002; Mosser and Edwards 2008; Mosser and Zhang 2008). It is possible that the inhibitory effect of B and 5α-THB on the formation of new vessels is explainable by a dual mode of action. By decreasing the proportion of classically-activated macrophages, the steroids may decrease inflammation and, thus, angiogenesis; on the other hand, by decreasing the alternatively-activated population, they may lower the release of pro-angiogenic mediators. It would be of interest, therefore, to investigate whether B and 5α-THB differentially affect the two groups of macrophages. One way would be to immuno-stain the alternatively-activated macrophages using an antibody against the antigen YM1, a protein that is specifically secreted by this population (Loke, Nair et al. 2002; Mosser and Edwards 2008). Interestingly, these cells do not convert the amino acid arginine into NO in order to kill bacteria, as done by classically-activated macrophages, but instead they transform it into ornithine, which is a precursor of collagen. This explains their participation in wound healing (Hesse, Modolell et al. 2001; Mosser and Zhang 2008). Understanding whether 5α-THB affects this population would also provide information about the likelihood that the compound may disrupt wound healing. To improve understanding of the cell types present in sponges, and how they are affected by steroids, more in-depth studies could be attempted. For instance, FACS analysis would be a useful tool to discern between different populations of inflammatory cells. However, this technique has not been extensively applied to this model, and optimisation studies may have to be performed beforehand. Measurement of the enzymatic activity of MPO and NAG (N-Acetylglutamate synthase), enzymes associated with active neutrophils or macrophages respectively, is an alternative method that has been widely used to quantify the two populations in a variety of implants in the sponge model of angiogenesis. This could be applied to the model presented here in future experiments.

The fact that 5α-THB, unlike B, did not decrease the amount of collagen in sponges, has potentially important functional consequences. Indeed, skin thinning induced by GCs is thought to be caused in part by reduced deposition of this protein in the

presence of topical steroids. Furthermore, and relevant to the angiogenesis model, this may be one of the reasons why the 5α-reduced compound is a weaker inhibitor of vessel growth than B, as the ECM plays a crucial role in the process by supporting and guiding the formation of the new vascular bed. Collagen production and deposition is also central in wound healing (Christian, Graham et al. 2006), suggesting that 5α-THB may not interfere with this process.

In the dermatitis model, 5α-THB had broader effects than in the model of angiogenesis; in effect, it regulated the abundance of transcripts for molecules involved in vascular permeability and many others. The intrinsic differences between the two models may account for the difference in behaviour. The dermatitis model represented an acute inflammatory response, whereas the sponge model represents a more complex setting where new tissue and vessels need to be built over a longer period of time. Experiments analysing shorter implantation times could shed more light on the role of 5α-THB in the early phases of the body response to the implants, when inflammation is particularly strong. Furthermore, *in vitro* studies, for example the ring aortic assay, ought to be a priority in order to investigate the mechanisms of action of 5α-THB in a more limited and controlled environment; indeed, this would allow testing of a wider range of concentrations of the compound, over a greater number of time points, and to quantify more easily a variety of soluble cytokines and factors involved in angiogenesis in response to 5α-THB. In addition, other *in vivo* models analysing angiogenesis in other contexts, such as wound healing, will have to be performed to gain a more comprehensive picture of the potential effects of 5α-THB on processes which are commonly disrupted by topically applied glucocorticoids, and are of clinical relevance.

The *in vitro* experiments analysing the trans-activation properties of 5α-THB also produced promising results, as the compound had very weak effects. More importantly, in the hepatic cell line BWTG3, 5α-THB did not increase the abundance of transcripts of any of the genes analysed. Interestingly, it antagonised some of the responses induced by B, being effective at concentrations as low as 10 and 100 nM

for the metabolic gene *Tat*. This may have important functional and pathophysiological consequences since both compounds are found in abundance in the liver, a major site where some of the adverse metabolic effects of GCs are triggered. In order to explain this phenomenon, GR does not necessarily need to be involved, as another receptor, LXRα, has been shown to repress GR transcriptional activity when activated both *in vivo* and *in vitro* in hepatoma cells (Nader, Ng et al. 2012). Indeed, whilst the experiments presented in the last chapter suggest the existence of an interaction between 5α-THB and GR, this needs to be confirmed. As mentioned above and in section 7.1, other receptors are interesting candidates and could explain the behaviour and selectivity of 5α-THB. One approach would be to perform chromatin immunoprecipitation analysis (ChIP), perhaps also combined with next-generation sequencing (ChIP-seq), in order to clarify if and to what extent the compound modifies the pattern of DNA sequences to which GR binds. However, these being expensive and elaborate methods, a good strategy may be to perform some preliminary experiments aimed to knock down GR in BWTG3 cells.

More experiments are needed to complete the work presented in this thesis, and to definitely verify the hypotheses presented. However, it can be concluded that 5α-THB is a new, promising, more selective, topical anti-inflammatory agent; indeed I have shown that: a) it is an effective anti-inflammatory compound *in vivo*, b) it is a weak inhibitor of angiogenesis and c) it weakly activates pathways linked with the onset of side effects.

I would like to conclude by highlighting one of the many things I have learnt from this work. I understood that the word “weaker” does not necessarily mean worse since, whatever causes this weakness, either the involvement of a different receptor than GR or a different interaction with GR itself, this characteristic may well be the reason behind 5α-THB being an effective and safer compound. Most importantly, I am excited that this thesis has opened new directions of research that we did not expect, and that is the beauty of scientific enquiry.

References

Abraham, S. M., T. Lawrence, et al. (2006). "Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1." J Exp Med 203(8): 1883-9.

Adams, M., O. C. Meijer, et al. (2003). "Homodimerization of the glucocorticoid receptor is not essential for response element binding: activation of the phenylethanolamine N-methyltransferase gene by dimerization-defective mutants." Mol Endocrinol 17(12): 2583-92.

Alangari, A. A. (2010). "Genomic and non-genomic actions of glucocorticoids in asthma." Ann Thorac Med 5(3): 133-9.

Andrade, S. P., T. P. Fan, et al. (1987). "Quantitative in-vivo studies on angiogenesis in a rat sponge model." Br J Exp Pathol 68(6): 755-66.

Andrew, R., D. I. Phillips, et al. (1998). "Obesity and gender influence cortisol secretion and metabolism in man." J Clin Endocrinol Metab 83(5): 1806-9.

Angeli, A., G. Guglielmi, et al. (2006). "High prevalence of asymptomatic vertebral fractures in post-menopausal women receiving chronic glucocorticoid therapy: a cross-sectional outpatient study." Bone 39(2): 253-9.

Antonakis, N., E. Markogiannakis, et al. (1991). "The antiglucocorticoid RU486 downregulates **the expression of interleukin-2 receptors in normal human lymphocytes." J Steroid Biochem Mol Biol 39(6): 929-35.**

Araujo, F. A., M. A. Rocha, et al. (2010). "Atorvastatin inhibits inflammatory angiogenesis in mice through down regulation of VEGF, TNF-alpha and TGF-beta1." Biomed Pharmacother 64(1): 29-34.

Armulik, A., G. Genove, et al. (2011). "Pericytes: developmental, physiological, and pathological perspectives, problems, and promises." Dev Cell 21(2): 193-215.

Aumais, J. P., H. S. Lee, et al. (1996). "Function of directly repeated half-sites as response elements for steroid hormone receptors." J Biol Chem 271(21): 12568-77.

Austyn, J. M. and S. Gordon (1981). "F4/80, a monoclonal antibody directed specifically against the mouse macrophage." Eur J Immunol 11(10): 805-15.

Avenant, C., A. Kotitschke, et al. (2010). "Glucocorticoid receptor phosphorylation modulates transcription efficacy through GRIP-1 recruitment." Biochemistry 49(5): 972-85.

Avenant, C., K. Ronacher, et al. (2010). "Role of ligand-dependent GR phosphorylation and half-life in determination of ligand-specific transcriptional activity." Mol Cell Endocrinol 327(1-2): 72-88.

Ayroldi, E., L. Cannarile, et al. (2012). "Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways." FASEB J 26(12): 4805-20.

Ayroldi, E., A. Macchiarulo, et al. (2014). "Targeting glucocorticoid side effects: selective glucocorticoid receptor modulator or glucocorticoid-induced leucine zipper? A perspective." FASEB J.

Ayush, O., C. H. Lee, et al. (2012). "Glutamine suppresses DNFB-induced contact dermatitis by deactivating p38 mitogen-activated protein kinase via induction of MAPK phosphatase-1." J Invest Dermatol 133(3): 723-31.

Aziz, K. E. and D. Wakefield (1996). "Modulation of endothelial cell expression of ICAM-1, E-selectin, and VCAM-1 by beta-estradiol, progesterone, and dexamethasone." Cell Immunol 167(1): 79-85.

Bae, C. J., S. B. Shim, et al. (2010). "IL-6, VEGF, KC and RANTES are a major cause of a high irritant dermatitis to phthalic anhydride in C57BL/6 inbred mice." Allergol Int 59(4): 389-97.

Banerjee, M., D. Robbins, et al. (2013). "Modulation of xenobiotic receptors by steroids." Molecules 18(7): 7389-406.

Barker, J. N., R. S. Mitra, et al. (1991). "Keratinocytes as initiators of inflammation." Lancet 337(8735): 211-4.

Barnes, P. J. (1998). "Anti-inflammatory actions of glucocorticoids: molecular mechanisms." Clin Sci (Lond) 94(6): 557-72.

Barnes, P. J. (2006). "Corticosteroids: the drugs to beat." Eur J Pharmacol 533(1-3): 2-14.

Barnes, P. J. (2006). "How corticosteroids control inflammation: Quintiles Prize Lecture 2005." Br J Pharmacol 148(3): 245-54.

Barnes, P. J. (2009). "Histone deacetylase-2 and airway disease." Ther Adv Respir Dis 3(5): 235-43.

Bartholome, B., C. M. Spies, et al. (2004). "Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis." FASEB J 18(1): 70-80.

Bartneck, M., F. M. Peters, et al. (2014). "Liposomal encapsulation of dexamethasone modulates cytotoxicity, inflammatory cytokine response, and migratory properties of primary human macrophages." Nanomedicine 10(6): 1209-20.

Baschant, U. and J. Tuckermann (2010). "The role of the glucocorticoid receptor in inflammation and immunity." J Steroid Biochem Mol Biol 120(2-3): 69-75.

Baumgartner, L., S. Sosa, et al. (2011). "Lignan derivatives from Krameria lappacea roots inhibit acute inflammation in vivo and pro-inflammatory mediators in vitro." J Nat Prod 74(8): 1779-86.

Beck, C. A., P. A. Estes, et al. (1993). "The steroid antagonist RU486 exerts different effects on the glucocorticoid and progesterone receptors." Endocrinology 133(2): 728-40.

Beck, C. A., N. L. Weigel, et al. (1993). "The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways." Proc Natl Acad Sci U S A 90(10): 4441-5.

Beck, I. M., Z. J. Drebert, et al. (2013). "Compound A, a selective glucocorticoid receptor modulator, enhances heat shock protein Hsp70 gene promoter activation." PLoS One 8(7): e69115.

Beer, H. D., R. Fassler, et al. (2000). "Glucocorticoid-regulated gene expression during cutaneous wound repair." Vitam Horm 59: 217-39.

Bellon, G., L. Martiny, et al. (2004). "Matrix metalloproteinases and matrikines in angiogenesis." Crit Rev Oncol Hematol 49(3): 203-20.

Belvisi, M. G., S. L. Wicks, et al. (2001). "Therapeutic benefit of a dissociated glucocorticoid and the relevance of in vitro separation of transrepression from transactivation activity." J Immunol 166(3): 1975-82.

Bentley, K., C. A. Franco, et al. (2014). "The role of differential VE-cadherin dynamics in cell rearrangement during angiogenesis." Nat Cell Biol 16(4): 309-21.

Bergers, G. and S. Song (2005). "The role of pericytes in blood-vessel formation and maintenance." Neuro Oncol 7(4): 452-64.

Bhattacharyya, S., D. E. Brown, et al. (2007). "Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase." Blood 109(10): 4313-9.

Biddie, S. C. (2011). "Chromatin architecture and the regulation of nuclear receptor inducible transcription." J Neuroendocrinol 23(1): 94-106.

Biddie, S. C., S. John, et al. (2011). "Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding." Mol Cell 43(1): 145-55.

Bir, S. C., Y. Xiong, et al. (2012). "Emerging role of PKA/eNOS pathway in therapeutic angiogenesis for ischaemic tissue diseases." Cardiovasc Res 95(1): 7-18.

Bladh, L. G., J. Liden, et al. (2005). "Identification of endogenous glucocorticoid repressed genes differentially regulated by a glucocorticoid receptor mutant able to separate between nuclear factor-kappaB and activator protein-1 repression." Mol Pharmacol 67(3): 815-26.

Blecharz, K. G., D. Drenckhahn, et al. (2008). "Glucocorticoids increase VE-cadherin expression and cause cytoskeletal rearrangements in murine brain endothelial cEND cells." J Cereb Blood Flow Metab 28(6): 1139-49.

Bledsoe, R. K., V. G. Montana, et al. (2002). "Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition." Cell 110(1): 93-105.

Blind, R. D. and M. J. Garabedian (2008). "Differential recruitment of glucocorticoid receptor phospho-isoforms to glucocorticoid-induced genes." J Steroid Biochem Mol Biol 109(1-2): 150-7.

Blomme, E. A., K. S. Chinn, et al. (2003). "Selective cyclooxygenase-2 inhibition does not affect the healing of cutaneous full-thickness incisional wounds in SKH-1 mice." Br J Dermatol 148(2): 211-23.

Bloomer, C. W., L. Kenyon, et al. (2003). "Cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) expression in human pituitary macroadenomas." Am J Clin Oncol 26(4): S75-80.

Blum, A. and E. Maser (2003). "Enzymology and molecular biology of glucocorticoid metabolism in humans." Prog Nucleic Acid Res Mol Biol 75: 173-216.

Brostjan, C., J. Anrather, et al. (1996). "Glucocorticoid-mediated repression of NFkappaB activity in endothelial cells does not involve induction of IkappaBalpha synthesis." J Biol Chem 271(32): 19612-6.

Bungard, C. J., G. D. Hartman, et al. (2011). "Discovery of selective glucocorticoid receptor modulator MK-5932." Bioorg Med Chem 19(24): 7374-86.

Carmeliet, P. and D. Collen (2000). "Molecular basis of angiogenesis. Role of VEGF and VE-cadherin." Ann N Y Acad Sci 902: 249-62; discussion 262-4.

Cavalcanti, D. M., C. M. Lotufo, et al. (2007). "Endogenous glucocorticoids control neutrophil mobilization from bone marrow to blood and tissues in non-inflammatory conditions." Br J Pharmacol 152(8): 1291-300.

Cavet, M., K. Harrington, et al. (2010). "Mapracorat, a novel selective glucocorticoid receptor agonist, inhibits hyperosmolar-induced cytokine release and MAPK pathways in human corneal epithelial cells." Molecular vision 16: 1791-1800.

Chang, K. C., Q. Shen, et al. (2008). "Liver X receptor is a therapeutic target for photoaging and chronological skin aging." Mol Endocrinol 22(11): 2407-19.

Chen, B. T., W. X. Li, et al. (2012). "Anti-inflammatory effects of a polyphenols-rich extract from tea (Camellia sinensis) flowers in acute and chronic mice models." Oxid Med Cell Longev 2012: 537923.

Chen, W., T. Dang, et al. (2008). "Glucocorticoid receptor phosphorylation differentially affects target gene expression." Mol Endocrinol 22(8): 1754-66.

Chien, C. H., J. N. Lai, et al. (2009). "Mifepristone acts as progesterone antagonist of non-genomic responses but inhibits phytohemagglutinin-induced proliferation in human T cells." Hum Reprod 24(8): 1968-75.

Chivers, J. E., W. Gong, et al. (2006). "Analysis of the dissociated steroid RU24858 does not exclude a role for inducible genes in the anti-inflammatory actions of glucocorticoids." Mol Pharmacol 70(6): 2084-95.

Christian, L. M., J. E. Graham, et al. (2006). "Stress and wound healing." Neuroimmunomodulation 13(5-6): 337-46.

Clark, A. R. (2003). "MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids?" J Endocrinol 178(1): 5-12.

Clark, A. R. (2007). "Anti-inflammatory functions of glucocorticoid-induced genes." Mol Cell Endocrinol 275(1-2): 79-97.

Clark, A. R., J. L. Dean, et al. (2003). "Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38." FEBS Lett 546(1): 37-44.

Cleasby, M. E., D. E. Livingstone, et al. (2003). "Is programming of glucocorticoid receptor expression by prenatal dexamethasone in the rat secondary to metabolic derangement in adulthood?" Eur J Endocrinol 148(1): 129-38.

Cobice, D. F., C. L. Mackay, et al. (2013). "Mass spectrometry imaging for dissecting steroid intracrinology within target tissues." Anal Chem 85(23): 11576-84.

Coghlan, M. J., P. B. Jacobson, et al. (2003). "A novel antiinflammatory maintains glucocorticoid efficacy with reduced side effects." Mol Endocrinol 17(5): 860-9.

Cole, T. J., J. A. Blendy, et al. (1995). "Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation." Genes Dev 9(13): 1608-21.

Connolly, D. T., D. M. Heuvelman, et al. (1989). "Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis." J Clin Invest 84(5): 1470-8.

Conway, E. M., D. Collen, et al. (2001). "Molecular mechanisms of blood vessel growth." Cardiovasc Res 49(3): 507-21.

Coruzzi, G., C. Pozzoli, et al. (2011). "Strain-dependent effects of the histamine Hâ„ receptor antagonist JNJ7777120 in a murine model of acute skin inflammation." Experimental dermatology 21(1): 32-37.

Cronstein, B. N., S. C. Kimmel, et al. (1992). "A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1." Proc Natl Acad Sci U S A 89(21): 9991-5.

Croxtall, J. D., Q. Choudhury, et al. (2000). "Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism." Br J Pharmacol 130(2): 289-98.

Crum, R., S. Szabo, et al. (1985). "A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment." Science 230(4732): 1375-8.

Cruz-Topete, D. and J. A. Cidlowski (2014). "One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids." Neuroimmunomodulation 22(1-2): 20-32.

da Silva, G. L., N. D. Sperotto, et al. (2013). "P2X7 receptor is required for neutrophil accumulation in a mouse model of irritant contact dermatitis." Exp Dermatol 22(3): 184-8.

Dahlman-Wright, K., H. Siltala-Roos, et al. (1990). "Protein-protein interactions facilitate DNA binding by the glucocorticoid receptor DNA-binding domain." J Biol Chem 265(23): 14030-5.

Dahlman-Wright, K., A. Wright, et al. (1991). "Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids." J Biol Chem 266(5): 3107-12.

Danielsen, M., J. P. Northrop, et al. (1986). "The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing and expression of wild-type and mutant receptor proteins." EMBO J 5(10): 2513-22.

Davis, G. E. (2010). "The development of the vasculature and its extracellular matrix: a gradual process defined by sequential cellular and matrix remodeling events." Am J Physiol Heart Circ Physiol 299(2): H245-7.

De Bosscher, K., I. M. Beck, et al. (2013). "Selective modulation of the glucocorticoid receptor can distinguish between transrepression of NF-kappaB and AP-1." Cell Mol Life Sci 71(1): 143-63.

De Bosscher, K., I. M. Beck, et al. "Classic glucocorticoids versus non-steroidal glucocorticoid receptor modulators: survival of the fittest regulator of the immune system?" Brain Behav Immun 24(7): 1035-42.

De Bosscher, K. and G. Haegeman (2009). "Minireview: latest perspectives on antiinflammatory actions of glucocorticoids." Mol Endocrinol 23(3): 281-91.

De Bosscher, K., M. L. Schmitz, et al. (1997). "Glucocorticoid-mediated repression of nuclear factor-kappaB-dependent transcription involves direct interference with transactivation." Proc Natl Acad Sci U S A 94(25): 13504-9.

De Bosscher, K., W. Vanden Berghe, et al. (2005). "A fully dissociated compound of plant origin for inflammatory gene repression." Proc Natl Acad Sci U S A 102(44): 15827-32.

De Bosscher, K., W. Vanden Berghe, et al. (2003). "The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression." Endocr Rev 24(4): 488-522.

de Lange, P., C. M. Segeren, et al. (2001). "Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells." Cancer Res 61(10): 3937-41.

Demerjian, M., E. H. Choi, et al. (2009). "Activators of PPARs and LXR decrease the adverse effects of exogenous glucocorticoids on the epidermis." Exp Dermatol 18(7): 643-9.

Dewint, P., V. Gossye, et al. (2008). "A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis." J Immunol 180(4): 2608-15.

Dhabhar, F. S. and B. S. McEwen (1999). "Enhancing versus suppressive effects of stress hormones on skin immune function." Proc Natl Acad Sci U S A 96(3): 1059-64.

Donet, E., P. Bosch, et al. (2008). "Transrepression function of the glucocorticoid receptor regulates eyelid development and keratinocyte proliferation but is not sufficient to prevent skin chronic inflammation." Mol Endocrinol 22(4): 799-812.

Doran, M. F., C. S. Crowson, et al. (2002). "Predictors of infection in rheumatoid arthritis." Arthritis Rheum 46(9): 2294-300.

Dressing, G. E., J. E. Goldberg, et al. (2010). "Membrane progesterone receptor expression in mammalian tissues: a review of regulation and physiological implications." Steroids 76(1-2): 11-7.

Drouin, J., Y. L. Sun, et al. (1993). "Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene." EMBO J 12(1): 145-56.

Duckles, S. P. and V. M. Miller (2010). "Hormonal modulation of endothelial NO production." Pflugers Arch 459(6): 841-51.

Dufour, A., E. Corsini, et al. (1998). "Modulation of ICAM-1, VCAM-1 and HLA-DR by cytokines and steroids on HUVECs and human brain endothelial cells." J Neurol Sci 157(2): 117-21.

Effendy, I., H. Loffler, et al. (2000). "Epidermal cytokines in murine cutaneous irritant responses." J Appl Toxicol 20(4): 335-41.

Evans, R. M. (1988). "The steroid and thyroid hormone receptor superfamily." Science 240(4854): 889-95.

Fang, H., R. A. Pengal, et al. (2004). "Lipopolysaccharide-induced macrophage inflammatory response is regulated by SHIP." J Immunol 173(1): 360-6.

Fardet, L., A. Flahault, et al. (2007). "Corticosteroid-induced clinical adverse events: frequency, risk factors and patient's opinion." Br J Dermatol 157(1): 142-8.

Fearnley, G. W., A. F. Odell, et al. (2014). "VEGF-A isoforms differentially regulate ATF-2-dependent VCAM-1 gene expression and endothelial-leukocyte interactions." Mol Biol Cell 25(16): 2509-21.

Ferreira, M. A., L. S. Barcelos, et al. (2004). "Sponge-induced angiogenesis and inflammation in PAF receptor-deficient mice (PAFR-KO)." Br J Pharmacol 141(7): 1185-92.

Fischer, S., D. Renz, et al. (2001). "In vitro effects of dexamethasone on hypoxia-induced hyperpermeability and expression of vascular endothelial growth factor." Eur J Pharmacol 411(3): 231-43.

Fitzpatrick, T. B., H. C. Griswold, et al. (1955). "Sodium retention and edema from percutaneous absorption of fludrocortisone acetate." J Am Med Assoc 158(13): 1149-52.

Folkman, J. and D. E. Ingber (1987). "Angiostatic steroids. Method of discovery and mechanism of action." Ann Surg 206(3): 374-83.

Folkman, J., R. Langer, et al. (1983). "Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone." Science 221(4612): 719-25.

Franklin, J., M. Lunt, et al. (2007). "Risk and predictors of infection leading to hospitalisation in a large primary-care-derived cohort of patients with inflammatory polyarthritis." Ann Rheum Dis 66(3): 308-12.

Fraser, R., M. C. Ingram, et al. (1999). "Cortisol effects on body mass, blood pressure, and cholesterol in the general population." Hypertension 33(6): 1364-8.

Freel, E. M. and J. M. Connell (2004). "Mechanisms of hypertension: the expanding role of aldosterone." J Am Soc Nephrol 15(8): 1993-2001.

Frijters, R., W. Fleuren, et al. (2010). "Prednisolone-induced differential gene expression in mouse liver carrying wild type or a dimerization-defective glucocorticoid receptor." BMC Genomics 11: 359.

Fukao, T. and S. Koyasu (2003). "PI3K and negative regulation of TLR signaling." Trends Immunol 24(7): 358-63.

Gahring, L. C., A. V. Osborne, et al. (2010). "Neuronal nicotinic alpha7 receptors modulate early neutrophil infiltration to sites of skin inflammation." J Neuroinflammation 7(1): 38.

Gaitan, D., C. R. DeBold, et al. (1995). "Glucocorticoid receptor structure and function in an adrenocorticotropin-secreting small cell lung cancer." Mol Endocrinol 9(9): 1193-201.

Galliher-Beckley, A. J., J. G. Williams, et al. (2011). "Ligand-Independent Phosphorylation of the Glucocorticoid Receptor Integrates Cellular Stress Pathways with Nuclear Receptor Signaling." Mol Cell Biol.

Gametchu, B., F. Chen, et al. (1999). "Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models." Steroids 64(1-2): 107-19.

Gao, Z., P. Chiao, et al. (2005). "Coactivators and corepressors of NF-kappaB in IkappaB alpha gene promoter." J Biol Chem 280(22): 21091-8.

Gerbal-Chaloin, S., I. Iankova, et al. (2013). "Nuclear receptors in the cross-talk of drug metabolism and inflammation." Drug Metab Rev 45(1): 122-44.

Gerhardt, H., M. Golding, et al. (2003). "VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia." J Cell Biol 161(6): 1163-77.

Goddard, L. M. and M. L. Iruela-Arispe (2013). "Cellular and molecular regulation of vascular permeability." Thromb Haemost 109(3): 407-15.

Gossye, V., D. Elewaut, et al. (2009). "Differential mechanism of NF-kappaB inhibition by two glucocorticoid receptor modulators in rheumatoid arthritis synovial fibroblasts." Arthritis Rheum 60(11): 3241-50.

Grabbe, S. and T. Schwarz (1998). "Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity." Immunol Today 19(1): 37-44.

Grange, T., J. Roux, et al. (1991). "Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor." Nucleic Acids Res 19(1): 131-9.

Gravanis, A., G. Schaison, et al. (1985). "Endometrial and pituitary responses to the steroidal antiprogestin RU 486 in postmenopausal women." J Clin Endocrinol Metab 60(1): 156-63.

Grose, R., S. Werner, et al. (2002). "A role for endogenous glucocorticoids in wound repair." EMBO Rep 3(6): 575-82.

Guess, A., S. Agrawal, et al. (2010). "Dose- and time-dependent glucocorticoid receptor signaling in podocytes." Am J Physiol Renal Physiol 299(4): F845-53.

Hadley, K. E., A. Louw, et al. (2011). "Differential nuclear localisation and promoter occupancy play a role in glucocorticoid receptor ligand-specific transcriptional responses." Steroids 76(10-11): 1176-84.

Hadoke, P. W., L. Macdonald, et al. (2006). "Intra-vascular glucocorticoid metabolism as a modulator of vascular structure and function." Cell Mol Life Sci 63(5): 565-78.

Hague, S., I. Z. MacKenzie, et al. (2002). "In-vivo angiogenesis and progestogens." Hum Reprod 17(3): 786-93.

Haller, J., E. Mikics, et al. (2008). "The effects of non-genomic glucocorticoid mechanisms on bodily functions and the central neural system. A critical evaluation of findings." Front Neuroendocrinol 29(2): 273-91.

Hard, T., K. Dahlman, et al. (1990). "Cooperativity and specificity in the interactions between DNA and the glucocorticoid receptor DNA-binding domain." Biochemistry 29(22): 5358-64.

Hasan, Q., S. T. Tan, et al. (2000). "Steroid therapy of a proliferating hemangioma: histochemical and molecular changes." Pediatrics 105(1 Pt 1): 117-20.

Hayden, M. S. and S. Ghosh (2008). "Shared principles in NF-kappaB signaling." Cell 132(3): 344-62.

Heasman, S. J., K. M. Giles, et al. (2003). "Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: implications for the resolution of inflammation." J Endocrinol 178(1): 29-36.

Hebeda, C. B., S. A. Teixeira, et al. (2008). "In vivo blockade of Ca(+2)-dependent nitric oxide synthases impairs expressions of L-selectin and PECAM-1." Biochem Biophys Res Commun 377(2): 694-8.

Hecker, E. (1967). "Phorbol esters from croton oil. Chemical nature and biological activities." Naturwissenschaften 54(11): 282-4.

Hengge, U. R., T. Ruzicka, et al. (2006). "Adverse effects of topical glucocorticosteroids." J Am Acad Dermatol 54(1): 1-15; quiz 16-8.

Herschman, H. R. (1994). "Regulation of prostaglandin synthase-1 and prostaglandin synthase-2." Cancer Metastasis Rev 13(3-4): 241-56.

Hesse, M., M. Modolell, et al. (2001). "Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism." J Immunol 167(11): 6533-44.

Higham, A., S. Lea, et al. (2014). "Corticosteroid effects on COPD alveolar macrophages: dependency on cell culture methodology." J Immunol Methods 405: 144-53.

Hoeben, A., B. Landuyt, et al. (2004). "Vascular endothelial growth factor and angiogenesis." Pharmacol Rev 56(4): 549-80.

Holmstrom, S., M. E. Van Antwerp, et al. (2003). "Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy." Proceedings of the National Academy of Sciences of the United States of America 100(26): 15758-15763.

Hong, C. and P. Tontonoz (2008). "Coordination of inflammation and metabolism by PPAR and LXR nuclear receptors." Curr Opin Genet Dev 18(5): 461-7.

Hoppe, U., P. M. Holterhus, et al. (2006). "Tissue-specific transcription profiles of sex steroid biosynthesis enzymes and the androgen receptor." J Mol Med (Berl) 84(8): 651-9.

Hori, Y., D. E. Hu, et al. (1996). "Differential effects of angiostatic steroids and dexamethasone on angiogenesis and cytokine levels in rat sponge implants." Br J Pharmacol 118(7): 1584-91.

Horwitz, K. B. (1985). "The antiprogestin RU38 486: receptor-mediated progestin versus antiprogestin actions screened in estrogen-insensitive T47Dco human breast cancer cells." Endocrinology 116(6): 2236-45.

Hovring, P. I., V. Matre, et al. (1999). "Transcription of the human thyrotropin-releasing hormone receptor gene-analysis of basal promoter elements and glucocorticoid response elements." Biochem Biophys Res Commun 257(3): 829-34.

Howell, K. R., A. Kutiyanawalla, et al. (2011). "Long-term continuous corticosterone treatment decreases VEGF receptor-2 expression in frontal cortex." PLoS One 6(5): e20198.

Huang, W. and C. K. Glass (2010). "Nuclear receptors and inflammation control: molecular mechanisms and pathophysiological relevance." Arterioscler Thromb Vasc Biol 30(8): 1542-9.

Huggenberger, R. and M. Detmar (2011). "The cutaneous vascular system in chronic skin inflammation." J Investig Dermatol Symp Proc 15(1): 24-32.

Humphrey, E. L., J. H. Williams, et al. (2006). "Effects of dissociated glucocorticoids on OPG and RANKL in osteoblastic cells." Bone 38(5): 652-61.

Illanes, J., A. Dabancens, et al. (2002). "Effects of betamethasone, sulindac and quinacrine drugs on the inflammatory neoangiogenesis response induced by polyurethane sponge implanted in mouse." Biol Res 35(3-4): 339-45.

Ingber, D. E., J. A. Madri, et al. (1986). "A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution." Endocrinology 119(4): 1768-75.

Inoue, T., D. Plieth, et al. (2005). "Antibodies against macrophages that overlap in specificity with fibroblasts." Kidney international 67(6): 2488-2493.

Ismaili, N. and M. J. Garabedian (2004). "Modulation of glucocorticoid receptor function via phosphorylation." Ann N Y Acad Sci 1024: 86-101.

Iwasaki, K., E. Mishima, et al. (1995). "Effect of RU 486 on the atrophogenic and antiinflammatory effects of glucocorticoids in skin." Journal of dermatological science 10(2): 151-158.

Jackson, D. E. (2003). "The unfolding tale of PECAM-1." FEBS Lett 540(1-3): 7-14.

Janka-Junttila, M., E. Moilanen, et al. (2006). "The glucocorticoid RU24858 does not distinguish between transrepression and transactivation in primary human eosinophils." J Inflamm (Lond) 3: 10.

Joanny, E., Q. Ding, et al. (2012). "Anti-inflammatory effects of selective glucocorticoid receptor modulators are partially dependent on up-regulation of dual specificity phosphatase 1." Br J Pharmacol 165(4b): 1124-36.

Kadmiel, M. and J. A. Cidlowski (2013). "Glucocorticoid receptor signaling in health and disease." Trends Pharmacol Sci 34(9): 518-30.

Kassel, O., A. Sancono, et al. (2001). "Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1." EMBO J 20(24): 7108-16.

Katychev, A., X. Wang, et al. (2003). "Glucocorticoid-induced apoptosis in CNS microvascular pericytes." Dev Neurosci 25(6): 436-46.

Kaur, M., J. E. Chivers, et al. (2008). "Long-acting beta2-adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human

airway epithelial and smooth muscle cells." Mol Pharmacol 73(1): 203-14.

Kawase, Y., T. Hoshino, et al. (2003). "Exacerbated and prolonged allergic and non-allergic inflammatory cutaneous reaction in mice with targeted interleukin-18 expression in the skin." J Invest Dermatol 121(3): 502-9.

Keller-Wood, M. E. and M. F. Dallman (1984). "Corticosteroid inhibition of ACTH secretion." Endocr Rev 5(1): 1-24.

Kelly, A., H. Bowen, et al. (2008). "The glucocorticoid receptor beta isoform can mediate transcriptional repression by recruiting histone deacetylases." J Allergy Clin Immunol 121(1): 203-208 e1.

Kenny, O. M., C. M. McCarthy, et al. (2013). "Anti-inflammatory properties of potato glycoalkaloids in stimulated Jurkat and Raw 264.7 mouse macrophages." Life Sci 92(13): 775-82.

Kim, S. H., H. K. Oh, et al. (2008). "In vivo hepatitis B virus-neutralizing activity of an anti-HBsAg humanized antibody in chimpanzees." Exp Mol Med 40(1): 145-9.

Kino, T., I. Manoli, et al. (2009). "Glucocorticoid receptor (GR) beta has intrinsic, GRalpha-independent transcriptional activity." Biochem Biophys Res Commun 381(4): 671-5.

Kino, T., Y. A. Su, et al. (2009). "Human glucocorticoid receptor isoform beta: recent understanding of its potential implications in physiology and pathophysiology." Cell Mol Life Sci 66(21): 3435-48.

Kleinman, H. K., J. Graf, et al. (1987). "Role of basement membranes in cell differentiation." Ann N Y Acad Sci 513: 134-45.

Klekamp, J. G., K. Jarzecka, et al. (1997). "Vascular endothelial growth factor is expressed in ovine pulmonary vascular smooth muscle cells in vitro and regulated by hypoxia and dexamethasone." Pediatr Res 42(6): 744-9.

Klose, A., P. Zigrino, et al. (2013). "Monocyte/macrophage MMP-14 modulates cell infiltration and T-cell attraction in contact dermatitis but not in murine wound healing." Am J Pathol 182(3): 755-64.

Kobayashi, K., Y. Tsubosaka, et al. (2013). "Prostaglandin D2-DP signaling promotes endothelial barrier function via the cAMP/PKA/Tiam1/Rac1 pathway." Arterioscler Thromb Vasc Biol 33(3): 565-71.

Komuves, L. G., M. Schmuth, et al. (2002). "Oxysterol stimulation of epidermal differentiation is mediated by liver X receptor-beta in murine epidermis." J Invest Dermatol 118(1): 25-34.

Koo, H. J., S. Lee, et al. (2004). "Geniposide, an anti-angiogenic compound from the fruits of Gardenia jasminoides." Planta Med 70(5): 467-9.

Koo, H. J., Y. S. Song, et al. (2004). "Antiinflammatory effects of genipin, an active principle of gardenia." Eur J Pharmacol 495(2-3): 201-8.

Kotelevtsev, Y., M. C. Holmes, et al. (1997). "11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress." Proc Natl Acad Sci U S A 94(26): 14924-9.

Kracht, M. and J. Saklatvala (2002). "Transcriptional and post-transcriptional control of gene expression in inflammation." Cytokine 20(3): 91-106.

Krett, N. L., S. Pillay, et al. (1995). "A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients." Cancer Res 55(13): 2727-9.

Ku, H. O., S. H. Jeong, et al. (2009). "Gene expression profiles and pathways in skin inflammation induced by three different sensitizers and an irritant." Toxicol Lett 190(2): 231-7.

Kucharz, E. J. (1988). "Hormonal control of collagen metabolism--Part I." Endocrinologie 26(2): 69-79.

Kyriakides, T. R., Y. H. Zhu, et al. (2001). "Altered extracellular matrix remodeling and angiogenesis in sponge granulomas of thrombospondin 2-null mice." Am J Pathol 159(4): 1255-62.

La Baer, J. and K. R. Yamamoto (1994). "Analysis of the DNA-binding affinity, sequence specificity and context dependence of the glucocorticoid receptor zinc finger region." J Mol Biol 239(5): 664-88.

Le Drean, Y., N. Mincheneau, et al. (2002). "Potentiation of glucocorticoid receptor transcriptional activity by sumoylation." Endocrinology 143(9): 3482-9.

Leahy, K. M., A. T. Koki, et al. (2000). "Role of cyclooxygenases in angiogenesis." Curr Med Chem 7(11): 1163-70.

Lee, H., M. Stieger, et al. (2013). "Cytokines and chemokines in irritant contact dermatitis." Mediators of inflammation 2013: 916497.

Lee, H. J., B. Oran, et al. (2006). "Steroid myopathy in patients with acute graft-versus-host disease treated with high-dose steroid therapy." Bone Marrow Transplant 38(4): 299-303.

Lefebvre, P., D. S. Berard, et al. (1991). "Two regions of the mouse mammary tumor virus long terminal repeat regulate the activity of its promoter in mammary cell lines." Mol Cell Biol 11(5): 2529-37.

Leibovich, S. J., P. J. Polverini, et al. (1987). "Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha." Nature 329(6140): 630-2.

Lewis-Tuffin, L. J., C. M. Jewell, et al. (2007). "Human glucocorticoid receptor beta binds RU-486 and is transcriptionally active." Mol Cell Biol 27(6): 2266-82.

Lim, H. Y., N. Muller, et al. (2007). "Glucocorticoids exert opposing effects on macrophage function dependent on their concentration." Immunology 122(1): 47-53.

Lim, W., C. Park, et al. (2014). "Glucocorticoids suppress hypoxia-induced COX-2 and hypoxia inducible factor-1alpha expression through the induction of glucocorticoid-induced leucine zipper." Br J Pharmacol 171(3): 735-45.

Livingstone, D. E., P. Barat, et al. (2014). "5alpha-Reductase type 1 deficiency or inhibition predisposes to insulin resistance, hepatic steatosis and liver fibrosis in rodents." Diabetes.

Livingstone, D. E., E. M. Di Rollo, et al. (2014). "Relative adrenal insufficiency in mice deficient in 5alpha-reductase 1." J Endocrinol 222(2): 257-66.

Livingstone, D. E., K. J. McInnes, et al. (2005). "Increased A-ring reduction of glucocorticoids in obese Zucker rats: effects of insulin sensitization." Obes Res 13(9): 1523-6.

Livingstone, D. E., C. Sykes, et al. (2014). 5alpha-Tetrahydrocorticosterone: A Novel Topical Anti-Inflammatory Agent with Improved Therapeutic Index. Glucocorticoid Actions: MON-0802-MON-0802.

Livingstone, D. E. W., C. Sykes, et al. (2014). "5alpha-Tetrahydrocorticosterone: A Novel Topical Anti-Inflammatory Agent with Improved Therapeutic Index." Endocrine Reviews 35(3): MON-0802-MON-0802.

Logie, J. J., S. Ali, et al. (2010). "Glucocorticoid-mediated inhibition of angiogenic changes in human endothelial cells is not caused by reductions in cell proliferation or migration." PLoS One 5(12): e14476.

Lohmann, C., M. Krischke, et al. (2004). "Tyrosine phosphatase inhibition induces loss of blood-brain barrier integrity by matrix metalloproteinase-dependent and -independent pathways." Brain Res 995(2): 184-96.

Loke, P., M. G. Nair, et al. (2002). "IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype." BMC Immunol 3: 7.

Longenecker, J. P., L. A. Kilty, et al. (1984). "Glucocorticoid inhibition of vascular smooth muscle cell proliferation: influence of homologous extracellular matrix and serum mitogens." J Cell Biol 98(2): 534-40.

Lopez, F. J., R. J. Ardecky, et al. (2008). "LGD-5552, an antiinflammatory glucocorticoid receptor ligand with reduced side effects, in vivo." Endocrinology 149(5): 2080-9.

Lowenberg, M., C. Stahn, et al. (2008). "Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands." Steroids 73(9-10): 1025-9.

Luo, M., X. Lu, et al. (2013). "A conserved protein motif is required for full modulatory activity of negative elongation factor subunits NELF-A and NELF-B in modifying glucocorticoid receptor-regulated gene induction properties." J Biol Chem 288(47): 34055-72.

Luu-The, V., G. Pelletier, et al. (2005). "Quantitative appreciation of steroidogenic gene expression in mouse tissues: new roles for type 2 5alpha-reductase, 20alpha-hydroxysteroid dehydrogenase and estrogen sulfotransferase." J Steroid Biochem Mol Biol 93(2-5): 269-76.

Lynch, J. T., R. Rajendran, et al. (2010). "The role of glucocorticoid receptor phosphorylation in Mcl-1 and NOXA gene expression." Mol Cancer 9: 38.

Mahendroo, M. S., K. M. Cala, et al. (1997). "Fetal death in mice lacking 5alpha-reductase type 1 caused by estrogen excess." Mol Endocrinol 11(7): 917-27.

Mahendroo, M. S., K. M. Cala, et al. (1996). "5 alpha-reduced androgens play a key role in murine parturition." Mol Endocrinol 10(4): 380-92.

Majewska, M. D. (1987). "Antagonist-type interaction of glucocorticoids with the GABA receptor-coupled chloride channel." Brain Res 418(2): 377-82.

Man, M. Q., G. D. Barish, et al. (2008). "Deficiency of PPARbeta/delta in the epidermis results in defective cutaneous permeability barrier

homeostasis and increased inflammation." J Invest Dermatol 128(2): 370-7.

Maragoudakis, M. E., M. Sarmonika, et al. (1989). "Antiangiogenic action of heparin plus cortisone is associated with decreased collagenous protein synthesis in the chick chorioallantoic membrane system." J Pharmacol Exp Ther 251(2): 679-82.

Masferrer, J. L. and K. Seibert (1994). "Regulation of prostaglandin synthesis by glucocorticoids." Receptor 4(1): 25-30.

McCurley, A., A. McGraw, et al. (2013). "Smooth muscle cell mineralocorticoid receptors: role in vascular function and contribution to cardiovascular disease." Pflugers Arch 465(12): 1661-70.

McDonnell, D. P., S. L. Dana, et al. (1995). "Cellular mechanisms which distinguish between hormone- and antihormone-activated estrogen receptor." Ann N Y Acad Sci 761: 121-37.

McDonough, A. K., J. R. Curtis, et al. (2008). "The epidemiology of glucocorticoid-associated adverse events." Curr Opin Rheumatol 20(2): 131-7.

McInnes, K. J., C. J. Kenyon, et al. (2004). "5alpha-reduced glucocorticoids, novel endogenous activators of the glucocorticoid receptor." J Biol Chem 279(22): 22908-12.

McKenzie, R. C. and D. N. Sauder (1990). "The role of keratinocyte cytokines in inflammation and immunity." J Invest Dermatol 95(6 Suppl): 105S-107S.

Meijer, O. C. (2002). "Coregulator proteins and corticosteroid action in the brain." J Neuroendocrinol 14(6): 499-505.

Meinel, S., M. Gekle, et al. (2014). "Mineralocorticoid receptor signaling: Crosstalk with membrane receptors and other modulators." Steroids.

Michailidou, Z. (2007). Modelling altered Glucocorticoid Sensitivity: From the HPA axis to metabolic abnormalities in mice and humans. Edinburgh, University of Edinburgh. Doctor of Philosophy.

Michailidou, Z., R. N. Carter, et al. (2008). "Glucocorticoid receptor haploinsufficiency causes hypertension and attenuates hypothalamic-pituitary-adrenal axis and blood pressure adaptions to high-fat diet." FASEB J 22(11): 3896-907.

Michalopoulos, G. K., W. C. Bowen, et al. (2003). "HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures." Gene Expr 11(2): 55-75.

Mo, J., P. Panichayupakaranant, et al. (2013). "Topical anti-inflammatory potential of standardized pomegranate rind extract and ellagic acid in contact dermatitis." Phytother Res 28(4): 629-32.

Moguilewsky, M. and D. Philibert (1984). "RU 38486: potent antiglucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation." J Steroid Biochem 20(1): 271-6.

Mohan, R. and R. A. Heyman (2003). "Orphan nuclear receptor modulators." Curr Top Med Chem 3(14): 1637-47.

Morgan, S. A., E. L. McCabe, et al. (2014). "11beta-HSD1 is the major regulator of the tissue-specific effects of circulating glucocorticoid excess." Proc Natl Acad Sci U S A 111(24): E2482-91.

Morimoto, H., M. Takahashi, et al. (2006). "Cardiac overexpression of monocyte chemoattractant protein-1 in transgenic mice prevents cardiac dysfunction and remodeling after myocardial infarction." Circ Res 99(8): 891-9.

Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol 8(12): 958-69.

Mosser, D. M. and X. Zhang (2008). "Activation of murine macrophages." Curr Protoc Immunol Chapter 14: Unit 14 2.

Moura, S. A., L. D. Lima, et al. (2011). "Local drug delivery system: inhibition of inflammatory angiogenesis in a murine sponge model by dexamethasone-loaded polyurethane implants." J Pharm Sci 100(7): 2886-95.

Muramoto, K., M. Goto, et al. (2010). "E6201, a novel kinase inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase-1: in vivo effects on cutaneous inflammatory responses by topical administration." J Pharmacol Exp Ther 335(1): 23-31.

Nader, N., S. S. Ng, et al. (2012). "Liver x receptors regulate the transcriptional activity of the glucocorticoid receptor: implications for the carbohydrate metabolism." PLoS One 7(3): e26751.

Nahrendorf, M., F. K. Swirski, et al. (2007). "The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions." J Exp Med 204(12): 3037-47.

Nauck, M., M. Roth, et al. (1997). "Induction of vascular endothelial growth factor by platelet-activating factor and platelet-derived growth factor is downregulated by corticosteroids." Am J Respir Cell Mol Biol 16(4): 398-406.

Nehls, V. and D. Drenckhahn (1993). "The versatility of microvascular pericytes: from mesenchyme to smooth muscle?" Histochemistry 99(1): 1-12.

Newman, A. C., M. N. Nakatsu, et al. (2011). "The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation." Mol Biol Cell 22(20): 3791-800.

Newton, R. (2000). "Molecular mechanisms of glucocorticoid action: what is important?" Thorax 55(7): 603-13.

Newton, R. and N. S. Holden (2007). "Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor?" Mol Pharmacol 72(4): 799-809.

Nguyen, P. N., I. Ross Young, et al. (2004). "Allopregnanolone in the brain and blood after disruption of the hypothalamic-pituitary-adrenal axis in fetal sheep." J Endocrinol 182(1): 81-8.

Nissen, R. M. and K. R. Yamamoto (2000). "The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the

RNA polymerase II carboxy-terminal domain." Genes Dev 14(18): 2314-29.

Nixon, M. (2011). Interactions between glucocorticoid metabolism and inflammation in obesity and insulin resistance, University of Edinburgh. Doctor of Philosophy.

Nixon, M., R. Upreti, et al. (2011). "5alpha-Reduced glucocorticoids: a story of natural selection." J Endocrinol 212(2): 111-27.

Nordeen, S. K., B. J. Suh, et al. (1990). "Structural determinants of a glucocorticoid receptor recognition element." Mol Endocrinol 4(12): 1866-73.

Novak, M. L. and T. J. Koh (2013). "Macrophage phenotypes during tissue repair." J Leukoc Biol 93(6): 875-81.

O'Neill, L. A. (2008). "'Fine tuning' TLR signaling." Nat Immunol 9(5): 459-61.

O'Neill, L. A. (2008). "Immunology. How frustration leads to inflammation." Science 320(5876): 619-20.

Oakley, R. H. and J. A. Cidlowski (2010). "Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids." J Biol Chem 286(5): 3177-84.

Oakley, R. H. and J. A. Cidlowski (2013). "The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease." J Allergy Clin Immunol 132(5): 1033-44.

Oishi, Y., Z. W. Fu, et al. (2002). "Molecular basis of the alteration in skin collagen metabolism in response to in vivo dexamethasone treatment: effects on the synthesis of collagen type I and III, collagenase, and tissue inhibitors of metalloproteinases." Br J Dermatol 147(5): 859-68.

Okada, H., S. Ban, et al. (2000). "Progressive renal fibrosis in murine polycystic kidney disease: an immunohistochemical observation." Kidney Int 58(2): 587-97.

Oliani, S. M., G. A. Ciocca, et al. (2008). "Fluctuation of annexin-A1 positive mast cells in chronic granulomatous inflammation." Inflamm Res 57(10): 450-6.

Orchinik, M., T. F. Murray, et al. (1991). "A corticosteroid receptor in neuronal membranes." Science 252(5014): 1848-51.

Owens, D. M. and S. M. Keyse (2007). "Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases." Oncogene 26(22): 3203-13.

Pacheco, N. R., C. Pinto Nde, et al. (2014). "Cecropia pachystachya: a species with expressive in vivo topical anti-inflammatory and in vitro antioxidant effects." Biomed Res Int 2014: 301294.

Peeters, B. W., G. S. Ruigt, et al. (2008). "Differential effects of the new glucocorticoid receptor antagonist ORG 34517 and RU486 (mifepristone) on glucocorticoid receptor nuclear translocation in the AtT20 cell line." Ann N Y Acad Sci 1148: 536-41.

Pepper, M. S. (2001). "Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis." Arterioscler Thromb Vasc Biol 21(7): 1104-17.

Perissi, V., K. Jepsen, et al. (2010). "Deconstructing repression: evolving models of co-repressor action." Nat Rev Genet 11(2): 109-23.

Perretti, M. and A. Ahluwalia (2000). "The microcirculation and inflammation: site of action for glucocorticoids." Microcirculation 7(3): 147-61.

Pinto, N., T. Morais, et al. (2010). "Topical anti-inflammatory potential of Physalin E from Physalis angulata on experimental dermatitis in mice." Phytomedicine : international journal of phytotherapy and phytopharmacology 17(10): 740-743.

Pitzalis, C., N. Pipitone, et al. (2002). "Regulation of leukocyte-endothelial interactions by glucocorticoids." Ann N Y Acad Sci 966: 108-18.

Plumb, J., L. Robinson, et al. (2013). "Evaluation of glucocorticoid receptor function in COPD lung macrophages using beclomethasone-17-monopropionate." PLoS One 8(5): e64257.

Poolman, T. M., S. N. Farrow, et al. (2013). "Pin1 promotes GR transactivation by enhancing recruitment to target genes." Nucleic Acids Res 41(18): 8515-25.

Pratt, W. B. and D. O. Toft (2003). "Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery." Exp Biol Med (Maywood) 228(2): 111-33.

Pross, C., M. M. Farooq, et al. (2002). "Dexamethasone inhibits vascular smooth muscle cell migration via modulation of matrix metalloproteinase activity." J Surg Res 102(2): 57-62.

Pugh, C. W. and P. J. Ratcliffe (2003). "Regulation of angiogenesis by hypoxia: role of the HIF system." Nat Med 9(6): 677-84.

Radke, P. W., C. Weber, et al. (2004). "Dexamethasone and restenosis after coronary stent implantation: new indication for an old drug?" Curr Pharm Des 10(4): 349-55.

Radoja, N., M. Komine, et al. (2000). "Novel mechanism of steroid action in skin through glucocorticoid receptor monomers." Molecular and cellular biology 20(12): 4328-4339.

Rae, M., A. Mohamad, et al. (2009). "Cortisol inactivation by 11beta-hydroxysteroid dehydrogenase-2 may enhance endometrial angiogenesis via reduced thrombospondin-1 in heavy menstruation." J Clin Endocrinol Metab 94(4): 1443-50.

Rapkin, A. J., M. Morgan, et al. (1997). "Progesterone metabolite allopregnanolone in women with premenstrual syndrome." Obstet Gynecol 90(5): 709-14.

Rask, E., T. Olsson, et al. (2001). "Tissue-specific dysregulation of cortisol metabolism in human obesity." J Clin Endocrinol Metab 86(3): 1418-21.

Rauch, A., S. Seitz, et al. (2010). "Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor." Cell Metab 11(6): 517-31.

Rauner, M., C. Goettsch, et al. (2010). "Dissociation of osteogenic and immunological effects by the selective glucocorticoid receptor agonist, compound A, in human bone marrow stromal cells." Endocrinology 152(1): 103-112.

Rauner, M., C. Goettsch, et al. "Dissociation of osteogenic and immunological effects by the selective glucocorticoid receptor agonist, compound A, in human bone marrow stromal cells." Endocrinology 152(1): 103-12.

Rehman, M. U., Y. Yoshihisa, et al. (2012). "The anti-inflammatory effects of platinum nanoparticles on the lipopolysaccharide-induced inflammatory response in RAW 264.7 macrophages." Inflamm Res 61(11): 1177-85.

Reichardt, H. M., K. H. Kaestner, et al. (1998). "DNA binding of the glucocorticoid receptor is not essential for survival." Cell 93(4): 531-41.

Revollo, J. R. and J. A. Cidlowski (2009). "Mechanisms generating diversity in glucocorticoid receptor signaling." Ann N Y Acad Sci 1179: 167-78.

Rhen, T. and J. A. Cidlowski (2005). "Antiinflammatory action of glucocorticoids--new mechanisms for old drugs." N Engl J Med 353(16): 1711-23.

Robertson, D. B. and H. I. Maibach (1982). "Topical corticosteroids." Int J Dermatol 21(2): 59-67.

Robertson, S., F. Allie-Reid, et al. "Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound a." J Biol Chem 285(11): 8061-75.

Robertson, S., F. Allie-Reid, et al. (2009). "Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound a." J Biol Chem 285(11): 8061-75.

Rogatsky, I., J. C. Wang, et al. (2003). "Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor." Proc Natl Acad Sci U S A 100(24): 13845-50.

Roozendaal, B., A. Hernandez, et al. (2010). "Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification." J Neurosci 30(14): 5037-46.

Roszak, A. W., Y. A. Lefebvre, et al. (1990). "Structural requirements for the binding of dexamethasone to nuclear envelopes and plasma membranes." J Steroid Biochem Mol Biol 37(2): 201-14.

Ryser, S., A. Massiha, et al. (2004). "Stimulated initiation of mitogen-activated protein kinase phosphatase-1 (MKP-1) gene transcription involves the synergistic action of multiple cis-acting elements in the proximal promoter." Biochem J 378(Pt 2): 473-84.

Sakamoto, N., N. G. Tanaka, et al. (1987). "Inhibitory effects of heparin plus cortisone acetate on endothelial cell growth both in cultures and in tumor masses." J Natl Cancer Inst 78(3): 581-5.

Salvador, E., S. Shityakov, et al. (2014). "Glucocorticoids and endothelial cell barrier function." Cell Tissue Res 355(3): 597-605.

Sanchis, A., L. Alba, et al. (2012). "Keratinocyte-targeted overexpression of the glucocorticoid receptor delays cutaneous wound healing." PLoS One 7(1): e29701.

Sarashina, H., Y. Tsubosaka, et al. (2013). "Opposing immunomodulatory roles of prostaglandin D2 during the progression of skin inflammation." J Immunol 192(1): 459-65.

Sartorius, C. A., S. D. Groshong, et al. (1994). "New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only

antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP." Cancer Res 54(14): 3868-77.

Sau, S. and R. Banerjee (2014). "Cationic lipid-conjugated dexamethasone as a selective antitumor agent." Eur J Med Chem 83: 433-47.

Schacke, H., W. D. Docke, et al. (2002). "Mechanisms involved in the side effects of glucocorticoids." Pharmacol Ther 96(1): 23-43.

Schacke, H., H. Rehwinkel, et al. (2005). "Dissociated glucocorticoid receptor ligands: compounds with an improved therapeutic index." Curr Opin Investig Drugs 6(5): 503-7.

Schacke, H., A. Schottelius, et al. (2004). "Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects." Proc Natl Acad Sci U S A 101(1): 227-32.

Schacke, H., T. M. Zollner, et al. (2009). "Characterization of ZK 245186, a novel, selective glucocorticoid receptor agonist for the topical treatment of inflammatory skin diseases." Br J Pharmacol 158(4): 1088-103.

Schilling, J. A., W. Joel, et al. (1959). "Wound healing: a comparative study of the histochemical changes in granulation tissue contained in stainless steel wire mesh and polyvinyl sponge cylinders." Surgery 46: 702-10.

Schmuth, M., V. Moosbrugger-Martinz, et al. (2014). "Role of PPAR, LXR, and PXR in epidermal homeostasis and inflammation." Biochim Biophys Acta 1841(3): 463-73.

Schoneveld, O. J., I. C. Gaemers, et al. (2004). "Mechanisms of glucocorticoid signalling." Biochim Biophys Acta 1680(2): 114-28.

Schulman, I. G. (2010). "Nuclear receptors as drug targets for metabolic disease." Adv Drug Deliv Rev 62(13): 1307-15.

Schulz, M., M. Eggert, et al. (2002). "RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding." J Biol Chem 277(29): 26238-43.

Seckl, J. R. and B. R. Walker (2001). "Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue-specific amplifier of glucocorticoid action." Endocrinology 142(4): 1371-6.

Segard-Maurel, I., K. Rajkowski, et al. (1996). "Glucocorticosteroid receptor dimerization investigated by analysis of receptor binding to glucocorticosteroid responsive elements using a monomer-dimer equilibrium model." Biochemistry 35(5): 1634-42.

Shafiee, A., C. Bucolo, et al. (2010). "In vivo ocular efficacy profile of mapracorat, a novel selective glucocorticoid receptor agonist, in rabbit models of ocular disease." Invest Ophthalmol Vis Sci 52(3): 1422-30.

Shikatani, E. A., A. Trifonova, et al. (2012). "Inhibition of proliferation, migration and proteolysis contribute to corticosterone-mediated inhibition of angiogenesis." PLoS One 7(10): e46625.

Shin, J. H., J. H. Ryu, et al. (2013). "Short-term heating reduces the anti-inflammatory effects of fresh raw garlic extracts on the LPS-induced production of NO and pro-inflammatory cytokines by downregulating allicin activity in RAW 264.7 macrophages." Food Chem Toxicol 58: 545-51.

Shin, S., S. S. Joo, et al. (2010). "Ethanol extract of Angelica gigas inhibits croton oil-induced inflammation by suppressing the cyclooxygenase - prostaglandin pathway." J Vet Sci 11(1): 43-50.

Siiteri, P. K. and J. D. Wilson (1974). "Testosterone formation and metabolism during male sexual differentiation in the human embryo." J Clin Endocrinol Metab 38(1): 113-25.

Small, G. R. (2006). Glucocorticoids and Angiogenesis. Edinburgh, University of Edinburgh. Doctor of Philosophy.

Small, G. R., P. W. Hadoke, et al. (2005). "Preventing local regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1 enhances angiogenesis." Proc Natl Acad Sci U S A 102(34): 12165-70.

Smoak, K. A. and J. A. Cidlowski (2004). "Mechanisms of glucocorticoid receptor signaling during inflammation." Mech Ageing Dev 125(10-11): 697-706.

So, A. Y., C. Chaivorapol, et al. (2007). "Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor." PLoS Genet 3(6): e94.

Song, I. H. and F. Buttgereit (2006). "Non-genomic glucocorticoid effects to provide the basis for new drug developments." Mol Cell Endocrinol 246(1-2): 142-6.

Song, Y., F. Zhao, et al. (2013). "Ginsenoside Rg1 exerts synergistic anti-inflammatory effects with low doses of glucocorticoids in vitro." Fitoterapia 91: 173-9.

Sottile, J. (2004). "Regulation of angiogenesis by extracellular matrix." Biochim Biophys Acta 1654(1): 13-22.

Stahn, C., M. Lowenberg, et al. (2007). "Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists." Mol Cell Endocrinol 275(1-2): 71-8.

Steinberg, K. P., L. D. Hudson, et al. (2006). "Efficacy and safety of corticosteroids for persistent acute respiratory distress syndrome." N Engl J Med 354(16): 1671-84.

Steinbuch, M., T. E. Youket, et al. (2004). "Oral glucocorticoid use is associated with an increased risk of fracture." Osteoporos Int 15(4): 323-8.

Stellacci, E., A. Di Noia, et al. (2009). "Interaction between the glucocorticoid and erythropoietin receptors in human erythroid cells." Exp Hematol 37(5): 559-72.

Stojadinovic, O., B. Lee, et al. (2007). "Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation." The Journal of biological chemistry 282(6): 4021-4034.

Stojadinovic, O., A. Sawaya, et al. (2013). "Glucocorticoid receptor localizes to adherens junctions at the plasma membrane of keratinocytes." PLoS One 8(4): e63453.

Stratis, A., M. Pasparakis, et al. (2006). "Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation." J Clin Invest 116(8): 2094-104.

Sulzberger, M. B. and V. H. Witten (1952). "The effect of topically applied compound F in selected dermatoses." J Invest Dermatol 19(2): 101-2.

Sunderkotter, C., T. Nikolic, et al. (2004). "Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response." J Immunol 172(7): 4410-7.

Szapary, D., L. N. Song, et al. (2008). "Differential modulation of glucocorticoid and progesterone receptor transactivation." Mol Cell Endocrinol 283(1-2): 114-26.

Tang, H., C. Schlapbach, et al. (2010). "Characterization of dendritic cells and macrophages in irritant contact dermatitis." J Dermatol Sci 57(3): 216-8.

Terao, M., M. Tani, et al. (2014). "11beta-hydroxysteroid dehydrogenase 1 specific inhibitor increased dermal collagen content and promotes fibroblast proliferation." PLoS One 9(3): e93051.

Thomas-Chollier, M., L. C. Watson, et al. (2013). "A naturally occurring insertion of a single amino acid rewires transcriptional regulation by glucocorticoid receptor isoforms." Proc Natl Acad Sci U S A 110(44): 17826-31.

Tian, H., Y. Matsuo, et al. (2013). "Thioredoxin ameliorates cutaneous inflammation by regulating the epithelial production and release of pro-inflammatory cytokines." Front Immunol 4: 269.

Tiberio, L., R. Nascimbeni, et al. (2013). "The decrease of mineralcorticoid receptor drives angiogenic pathways in colorectal cancer." PLoS One 8(3): e59410.

Tiganescu, A., M. Hupe, et al. (2014). "Increased glucocorticoid activation during mouse skin wound healing." J Endocrinol 221(1): 51-61.

Tiganescu, A., A. A. Tahrani, et al. (2013). "11beta-Hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects." J Clin Invest 123(7): 3051-60.

Tiganescu, A., E. A. Walker, et al. (2011). "Localization, age- and site-dependent expression, and regulation of 11beta-hydroxysteroid dehydrogenase type 1 in skin." J Invest Dermatol 131(1): 30-6.

Tonelli, G., L. Thibault, et al. (1965). "A bio-assay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticoids." Endocrinology 77(4): 625-34.

Towbin, H., W. Pignat, et al. (1995). "Time-dependent cytokine production in the croton oil-induced mouse ear oedema and inhibition by prednisolone." Inflamm Res 44 Suppl 2: S160-1.

Tragni, E., A. Tubaro, et al. (1985). "Evidence from two classic irritation tests for an anti-inflammatory action of a natural extract, Echinacina B." Food Chem Toxicol 23(2): 317-9.

Trebble, P. J., J. M. Woolven, et al. (2013). "A ligand-specific kinetic switch regulates glucocorticoid receptor trafficking and function." J Cell Sci 126(Pt 14): 3159-69.

Tsai, S. Y., J. Carlstedt-Duke, et al. (1988). "Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation." Cell 55(2): 361-9.

Tsunemi, Y., H. Saeki, et al. (2006). "CCL17 transgenic mice show an enhanced Th2-type response to both allergic and non-allergic stimuli." Eur J Immunol 36(8): 2116-27.

Tubaro, A., P. Dri, et al. (1986). "The croton oil ear test revisited." Agents Actions 17(3-4): 347-9.

Tuckermann, J. P., H. M. Reichardt, et al. (1999). "The DNA binding-independent function of the glucocorticoid receptor mediates repression of AP-1-dependent genes in skin." J Cell Biol 147(7): 1365-70.

Uhlenhaut, N. H., G. D. Barish, et al. (2013). "Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes." Mol Cell 49(1): 158-71.

Unterberger, C., K. J. Staples, et al. (2008). "Role of STAT3 in glucocorticoid-induced expression of the human IL-10 gene." Mol Immunol 45(11): 3230-7.

van Loo, G., M. Sze, et al. (2010). "Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis." Mol Endocrinol 24(2): 310-22.

van Staa, T. P., H. G. Leufkens, et al. (2000). "Use of oral corticosteroids in the United Kingdom." QJM 93(2): 105-11.

Vandevyver, S., L. Dejager, et al. (2013). "New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation." Endocrinology 154(3): 993-1007.

Vandevyver, S., L. Dejager, et al. (2012). "Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation." J Clin Invest 122(6): 2130-40.

Vassallo, A., N. De Tommasi, et al. (2013). "Steroids with anti-inflammatory activity from Vernonia nigritiana Oliv. & Hiern." Phytochemistry 96: 288-98.

Vayssiere, B. M., S. Dupont, et al. (1997). "Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo." Mol Endocrinol 11(9): 1245-55.

Versaci, F., A. Gaspardone, et al. (2002). "Immunosuppressive Therapy for the Prevention of Restenosis after Coronary Artery Stent Implantation (IMPRESS Study)." J Am Coll Cardiol 40(11): 1935-42.

Vilasco, M., L. Communal, et al. (2011). "Glucocorticoid receptor and breast cancer." Breast Cancer Res Treat 130(1): 1-10.

Visser, K., C. Smith, et al. (2010). "Interplay of the inflammatory and stress systems in a hepatic cell line: interactions between glucocorticoid receptor agonists and interleukin-6." Endocrinology 151(11): 5279-93.

Walker, B. R. (2007). "Glucocorticoids and cardiovascular disease." Eur J Endocrinol 157(5): 545-59.

Wallerath, T., K. Witte, et al. (1999). "Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension." Proc Natl Acad Sci U S A 96(23): 13357-62.

Wang, H., T. Peters, et al. (2006). "Activated macrophages are essential in a murine model for T cell-mediated chronic psoriasiform skin inflammation." J Clin Invest 116(8): 2105-14.

Wang, Z., J. Frederick, et al. (2002). "Deciphering the phosphorylation "code" of the glucocorticoid receptor in vivo." J Biol Chem 277(29): 26573-80.

Webster, J. C., C. M. Jewell, et al. (1997). "Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein." J Biol Chem 272(14): 9287-93.

Wei, L., T. M. MacDonald, et al. (2004). "Taking glucocorticoids by prescription is associated with subsequent cardiovascular disease." Ann Intern Med 141(10): 764-70.

Weischenfeldt, J. and B. Porse (2008). "Bone Marrow-Derived Macrophages (BMM): Isolation and Applications." CSH protocols 2008.

Welti, J., S. Loges, et al. (2013). "Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer." J Clin Invest 123(8): 3190-200.

Wen, F. Q., X. Liu, et al. (2003). "TH2 Cytokine-enhanced and TGF-beta-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN-gamma and corticosteroids." J Allergy Clin Immunol 111(6): 1307-18.

Wissink, S., A. van de Stolpe, et al. (1997). "NF-kappa B/Rel family members regulating the ICAM-1 promoter in monocytic THP-1 cells." Immunobiology 198(1-3): 50-64.

Wust, S., D. Tischner, et al. (2009). "Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis." PLoS One 4(12): e8202.

Xavier, D. O., L. S. Amaral, et al. (2010). "Metformin inhibits inflammatory angiogenesis in a murine sponge model." Biomed Pharmacother 64(3): 220-5.

Xin, W., L. Zhang, et al. (2011). "Escin exerts synergistic anti-inflammatory effects with low doses of glucocorticoids in vivo and in vitro." Phytomedicine 18(4): 272-7.

Yang, C. (2009). Investigation of the cellular and physiological effects of 5α-tetrahydro reduced glucocorticoids *in vitro* and *in vivo*, University of Edinburgh. Doctor of Philosophy.

Yang, C., M. Nixon, et al. (2011). "5alpha-reduced glucocorticoids exhibit dissociated anti-inflammatory and metabolic effects." Br J Pharmacol 164(6): 1661-71.

Yang, J. and P. J. Fuller (2012). "Interactions of the mineralocorticoid receptor--within and without." Mol Cell Endocrinol 350(2): 196-205.

Yang, N., W. Zhang, et al. (2008). "Glucocorticoid-induced leucine zipper (GILZ) mediates glucocorticoid action and inhibits inflammatory cytokine-induced COX-2 expression." J Cell Biochem 103(6): 1760-71.

Yau, J. L. and J. R. Seckl (2012). "Local amplification of glucocorticoids in the aging brain and impaired spatial memory." Front Aging Neurosci 4: 24.

Yossuck, P., Y. Yan, et al. (2001). "Dexamethasone alters TNF-alpha expression in retinopathy." Mol Genet Metab 72(2): 164-7.

Zeng, X., J. Zheng, et al. (2013). "A newly synthesized sinapic acid derivative inhibits endothelial activation in vitro and in vivo." Mol Pharmacol 83(5): 1099-108.

Zetterstrom, R. (2008). "The discovery that cortisone may effectively ameliorate inflammatory and allergic diseases." Acta Paediatr 97(4): 513-7.

Zhang, N., Z. Fang, et al. (2004). "Tracking angiogenesis induced by skin wounding and contact hypersensitivity using a Vegfr2-luciferase transgenic mouse." Blood 103(2): 617-26.

Zhang, S., J. Jonklaas, et al. (2007). "The glucocorticoid agonist activities of mifepristone (RU486) and progesterone are dependent on glucocorticoid receptor levels but not on EC50 values." Steroids 72(6-7): 600-8.

Zhang, X., A. F. Clark, et al. (2008). "FK506-binding protein 51 regulates nuclear transport of the glucocorticoid receptor beta and glucocorticoid responsiveness." Invest Ophthalmol Vis Sci 49(3): 1037-47.

Zhong, H. J., H. Y. Wang, et al. (2013). "Low concentrations of corticosterone exert stimulatory effects on macrophage function in a manner dependent on glucocorticoid receptors." Int J Endocrinol 2013: 405127.

Zhu, B. T. (2005). "Mechanistic explanation for the unique pharmacologic properties of receptor partial agonists." Biomed Pharmacother 59(3): 76-89.

Zimmermann, G. R., W. Avery, et al. (2009). "Selective amplification of glucocorticoid anti-inflammatory activity through synergistic multi-target action of a combination drug." Arthritis Res Ther 11(1): R12.

Zuckerman, S. H., G. F. Evans, et al. (1991). "Transcriptional and post-transcriptional mechanisms involved in the differential expression of LPS-induced IL-1 and TNF mRNA." Immunology 73(4): 460-5.